Results and Discussion of STR Analysis by Mass Spectrometry

In the course of this work, thousands of data points were collected using STR markers of forensic interest verifying that GeneTrace's mass spectrometry technology works. During this same time, tens of thousands of data points were gathered across hundreds of different microsatellite markers from corn and soybean as part of an ongoing plant genomics partnership with Monsanto Company (St. Louis, MO). Whether the DNA markers used come from humans or plants, the characteristics described below apply when analyzing polymorphic repeat loci.

Marker Selection and Feasibility Studies With STR Loci

Prior to receiving grant funding, feasibility work had been completed using the STR markers TH01, CSF1PO, FES/FPS, and F13A1 in the summer and fall of 1996 (Becker et al., 1997). At the start of this project, a number of STR loci were considered as possible candidates to expand upon the initial four STR markers and to develop a set of markers that would work well in the mass spectrometer and would be acceptable to the forensic DNA community. Searches were made of publicly available databases, including the Cooperative Human Linkage Center (*http://lpg.nci.nih.gov/CHLC*), the Genome Database (*http://gdbwww.gdb.org*), and Weber set 8 of the Marshfield Medical Research Foundation's Center for Medical Genetics (*http://www.marshmed.org/genetics*). Literature was also searched for possible tetranucleotide markers with PCR product sizes below 140 bp in size to avoid having to redesign the PCR primers to meet our limited size range needs (Hammond et al., 1994; Lindqvist et al., 1996). The desired characteristics also included high heterozygosity, moderate number of alleles (<7 or 8 to maintain a narrow mass range) with no known microvariants (to avoid the need for a high degree of resolution), and balanced allele frequencies (most commonly allele <40% and least common allele >5%). This type of marker screen was found to be rather inefficient because the original primer sets reported in public STR databases were designed for gel-based separations, which were optimal over a size range of 100–400 bp. In fact, most of the PCR product sizes were in the 200–300 bp range. From a set of several thousand publicly available STRs, only a set of eight candidate tetranucleotide STRs were initially identified; three of which were tested using the original reported primers (exhibit 33). The initial goal was to identify ~25 markers that spanned all 22 autosomal chromosomes as well as the X and Y sex chromosomes.

Researchers quickly realized that population data were not available on these "new" markers and would not be readily accepted without extensive testing and validation. Since one of the objectives was to produce STR marker sets that would be of value to the forensic DNA community, the next step taken was the examination of STR markers already in use. After selecting

Exhibit 33. **Tetranucleotide markers identified through literature and public database searches as possible candidates for early STR marker development.** The databases searched included the Cooperative Human Linkage Center, Marshfield Clinic Weber set 8, and the Genome Database. Primers were synthesized and tested for the markers in **bold**.

Marker Name	Heterozygosity	Size Range	Allele Frequencies	Number of Alleles	GenBank Sequence
D1S1612	0.83	94–134 bp	0.9–26%	10 alleles	G07863
D2S1391	0.79	109–137 bp	5–35%	8 alleles	G08168
D5S1457	0.74	97–127 bp	1–32%	8 alleles	G08431
GATA132B04 (D5S2843)	Not reported	98–114 bp	12–35%	5 alleles	G10407
D16S2622	Not reported	71–91 bp	3–54%	5 alleles	G07934
D16S764	0.70	96–116 bp	4–38%	5 alleles	G07928
D19S591	0.74	96–112 bp	6–36%	5 alleles	G09745
D22S445	0.65	110–130 bp	3–37%	6 alleles	G08096

STR markers used by the Promega Corporation, Applied Biosystems, and the Forensic Science Service (FSS), researchers redesigned primer pairs for each STR locus to produce smaller PCR product sizes. These STR markers included TPOX, D5S818, D7S820, D13S317, D16S539, LPL, F13B, HPRTB, D3S1358, VWA, FGA, CD4, D8S1179, D18S51, and D21S11. The primers for TH01 and CSF1PO were also redesigned to improve PCR efficiencies and to reduce the amplicon sizes. Primers for amelogenin, a commonly used sex-typing marker, were also tested (Sullivan et al., 1993). In addition, two Y-chromosome STRs, DYS19 and DYS391, were examined briefly. Exhibit 34 summarizes the STR primer sets that were developed and tested over the course of this project. However, with the announcement of the 13 CODIS core loci in the fall of 1997, emphasis switched to CSF1PO, TPOX, TH01, D3S1358, VWA, FGA, D5S818, D7S820, D13S317, D16S539, D8S1179, D18S51, D21S11, and the sex-typing marker amelogenin.

The newly designed GeneTrace primers produced smaller PCR products than those commercially available from Applied Biosystems or Promega (exhibit 2), yet resulted in identical genotypes in almost all samples tested. For example, correct genotypes were obtained on the human cell line K562, a commonly used control for PCR amplification success. Exhibit 35 shows the K562 results for CSF1PO, TPOX, TH01, and amelogenin. These results were included as part of a publication demonstrating that time-of-flight mass spectrometry could perform accurate genotyping of STRs without allelic ladders (Butler et al., 1998).

Exhibit 34. **STR markers examined at GeneTrace during the course of this project as sorted by their chromosomal position.** Primers were designed, synthesized, and tested for each of these markers. The most extensive testing was performed with the markers highlighted in green. Amelogenin, which is a gender identification marker rather than an STR, is listed twice because it occurs on both the X and Y chromosomes. The *italicized* STRs are those not commonly used by the forensic DNA community.

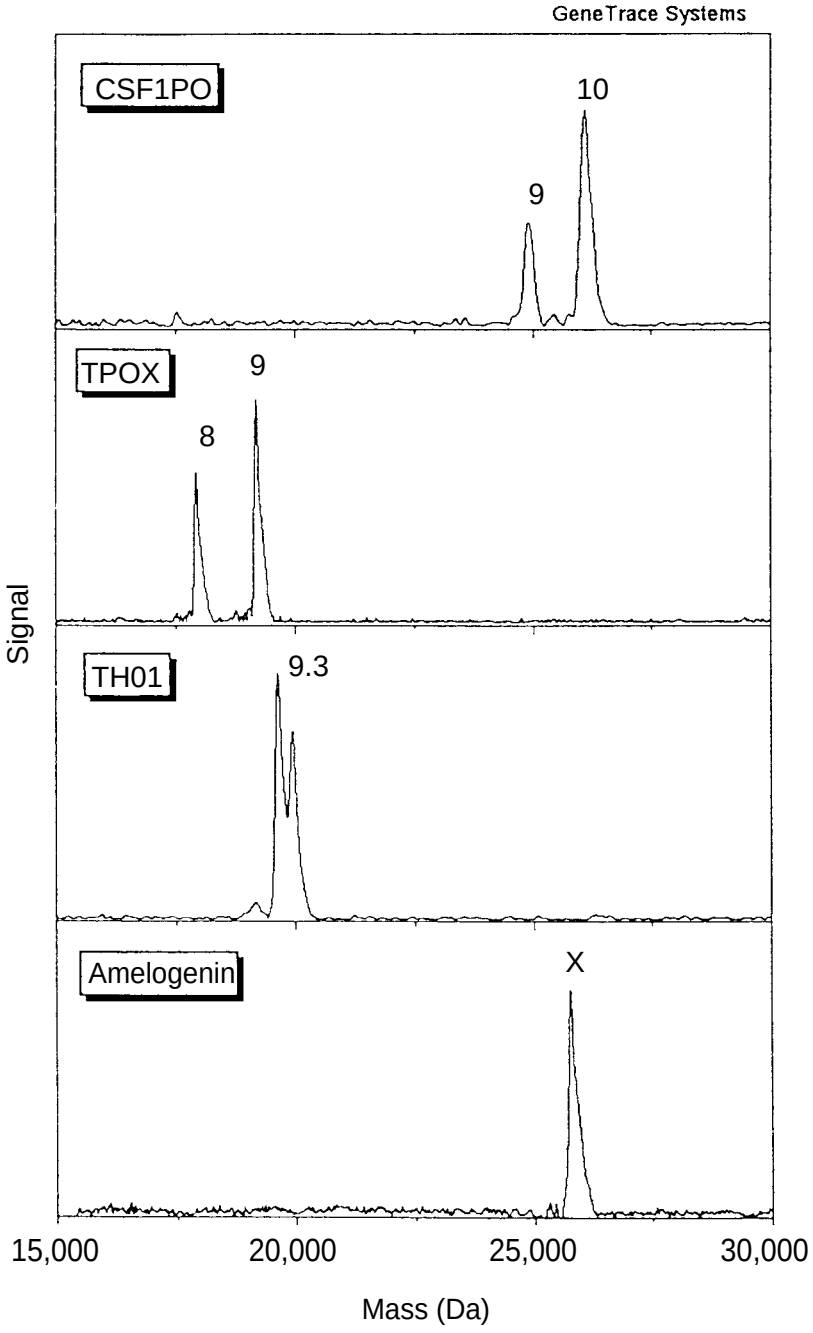
Human Chromosome	STR Marker	Human Chromosome	STR Marker
1	F13B	**13**	D13S317
2	**TPOX**	**14**	
3	**D3S1358**	**15**	FES/FPS
4	**FGA**	**16**	**D16S539,** *D16S2622*
5	**CSF1PO,** D5S818, *GATA132B04*	**17**	
6	F13A1	**18**	D18S51
7	**D7S820**	**19**	
8	LPL, **D8S1179**	**20**	
9		**21**	D21S11
10		**22**	*D22S445*
11	**TH01**	**X**	HPRTB, **Amelogenin**
12	VWA, CD4	**Y**	DYS19, **DYS391**, **Amelogenin**

Caveats of STR analysis by mass spectrometry

While mass spectrometry worked well for a majority of the STR markers tested, a few limitations excluded some STRs from working effectively. Two important issues that impact mass spectrometry results are DNA size and sample salts. Mass spectrometry resolution and sensitivity are diminished when either the DNA size or the salt content of the sample is too large (Ross and Belgrader, 1997; and Taranenko et al., 1998). By designing the PCR primers to bind close to the repeat region, the STR allele sizes are reduced so that resolution and sensitivity of the PCR products are benefited. In addition, the GeneTrace-patented cleavage step reduces the measured DNA size even further. When possible, primers are designed to produce amplicons that are less than 120 bp, although work is sometimes undertaken with STR alleles that are as large as 140 bp in size. This limitation in size prevents reliable analysis of STR markers with samples containing a large number of repeats, such as most of the FGA, D21S11, and D18S51 alleles (exhibit 2).

To overcome the sample salt problem, researchers used a patented solid-phase purification procedure that reduced the concentration of magnesium, potassium, and sodium salts in the PCR products prior to being introduced to the mass spectrometer (Monforte et al., 1997). Without the reduction of the salts, resolution is diminished by the presence of adducts. Salt molecules bind to the DNA during the MALDI ionization process and give rise to peaks that have a mass of the DNA molecule plus the salt molecule. Adducts broaden peaks and thus reduce peak resolution. The sample

Exhibit 35. **Mass spectra for CSF1PO, TPOX, TH01, and amelogenin using K562 DNA.** Genotypes agree with results reported by the manufacturer (Promega Corporation). The numbers above the peaks represent the allele calls based upon the observed mass. The allele imbalance on the heterozygous samples is because the K562 strain is known to contain an unusual number of chromosomes and some of them are represented more than twice per cell. The TH01 peak is split because it is not fully adenylated (Butler et al., 1998).



purification procedure, which was entirely automated on a 96-tip robotic workstation, reduced the PCR buffer salts and yielded "clean" DNA for the mass spectrometer. Appropriate care must be taken to prevent samples from being contaminated with salts both during and after the sample purification procedure.

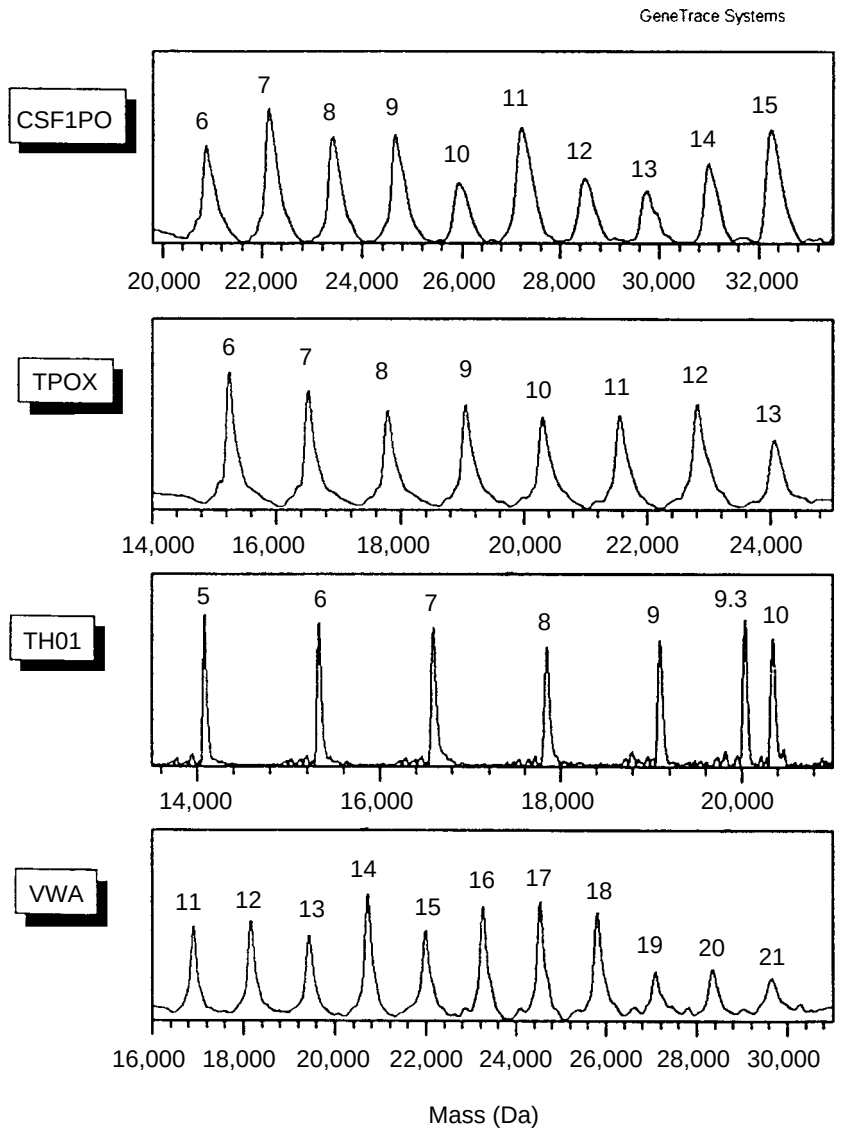
Size reduction methods

The portion of the DNA product on the other side of the repeat region from the cleavable primer was removed in one of two possible ways: using a restriction enzyme (Monforte et al., 1999) or performing a nested linear amplification with a ddN terminating nucleotide (Braun et al., 1997a and 1997b). Both methods have pros and cons. A restriction enzyme, *Dpn*II, which recognizes the sequence 5'... ^GATC...3', was used with VWA samples to remove 45 bp from each PCR product. For example, the GenBank allele that contains 18 repeat units and is 154 bp following PCR amplification may be reduced to 126 nucleotides following primer cleavage, but it can be shortened to 81 nucleotides if primer cleavage is combined with *Dpn*II digestion. At 81 nt or 25,482 Da, the STR product size is much more manageable in the mass spectrometer. This approach works nicely provided the restriction enzyme recognition site remains unchanged. The *Dpn*II digestion of VWA amplicons worked on all samples tested, including a reamplification of an allelic ladder from ABI (exhibit 36). However, the cost and time of analysis are increased with the addition of a restriction enzyme step.

The second approach for reducing the overall size of the DNA molecule in the mass spectrometer involved using a single ddNTP with three regular dNTPs. A linear amplification extension reaction was performed with the ddNTP terminating the reaction on the opposite side of the repeat from the cleavable primer. However, there

were several limitations with this "single base sequencing" approach. First, it only worked if the repeat did not contain all four nucleotides. For example, a nucleotide mixture of dideoxycytosine (ddC), deoxyadenosine (dA), deoxythymidine (dT), and deoxyguanosine (dG) will allow extension through an AATG repeat (as occurs in the bottom strand of TH01) but will terminate at the first C nucleotide in a TCAT repeat (the top strand of TH01). Thus one is limited with the DNA strand that can be used for a given combination of dideoxynucleotide and corresponding deoxynucleotides. In addition, primer position and STR sequence content are important. If a ddC mix is used, the DNA sample cannot contain any C nucleotides prior to the repeat region or within the repeat, or the extension will prematurely halt and the information content of the full repeat will not be accurately captured. In most cases, this requires the extension primer to be immediately adjacent to the STR repeat, a situation that is not universally available due to the flanking sequences around the repeat region. For example, this approach will work with TH01 (AATG) but not VWA, which has three different repeat structures: AGAT, AGAC, and AGGT. Thus with VWA, a ddC would extend through the AGAT repeat but would be prematurely terminated at the C in the AGAC repeat, and valuable polymorphic information would be lost.

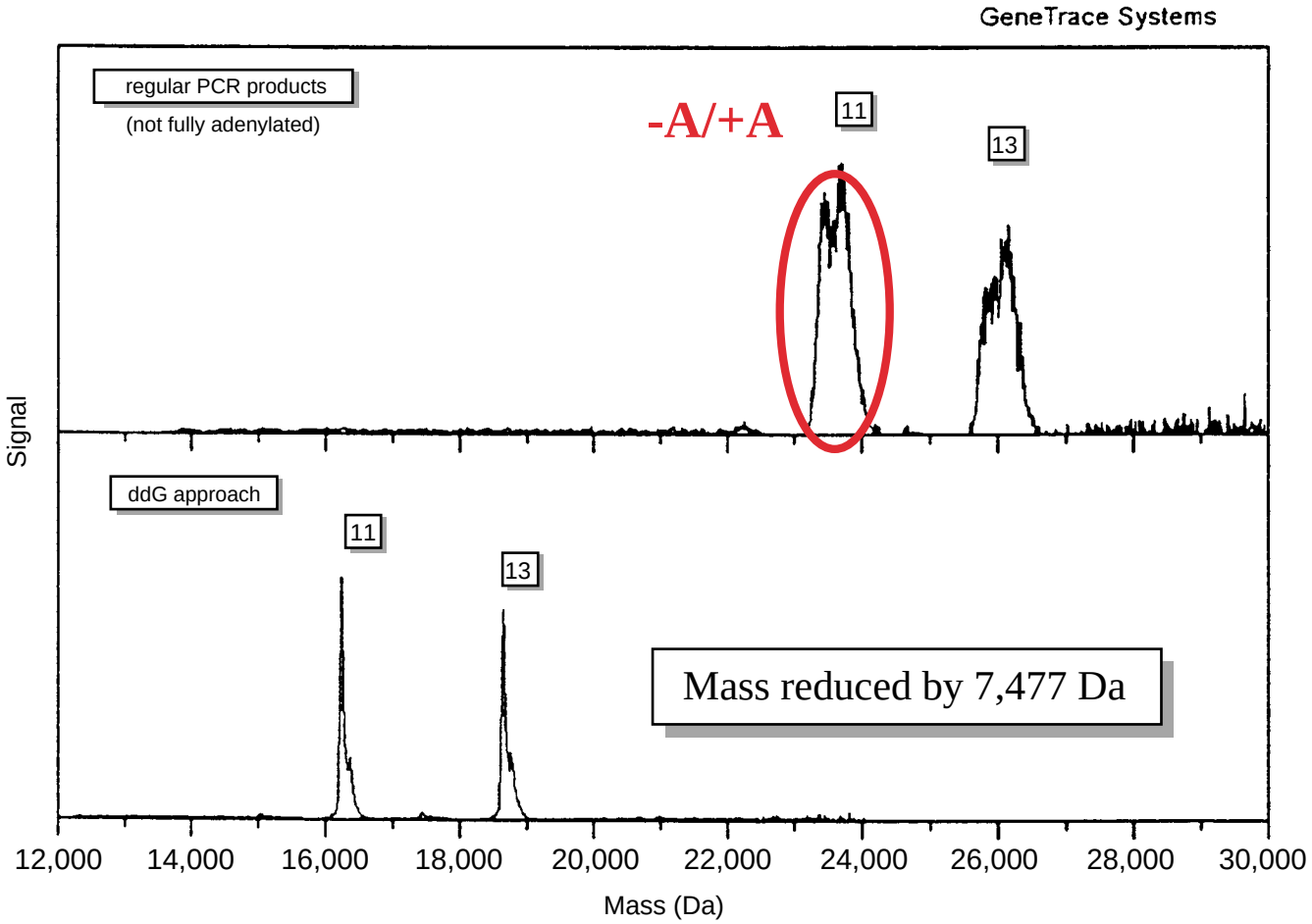
Exhibit 36. **Mass spectra of STR allelic ladders from CSF1PO, TPOX, TH01, and VWA.** The numbers above each peak designate the allele name (number of repeats). Peak widths vary among samples based on DNA size and salt content. Smaller sizes (e.g., TH01) give sharper peaks than larger sizes (e.g., CSF1PO). On a mass scale as shown here, each nucleotide is approximately 300 Daltons (Da). The VWA ladder was digested with *Dpn*II restriction enzyme following PCR to reduce the overall size of the amplicons.



The use of a terminating nucleotide also provides a sharper peak for an amplified allele compared with the split peaks or wider peaks (if resolution is poor) that can result from partially adenylated amplicons (i.e., -A/+A). Exhibit 37 illustrates the advantage of a ddG termination on a D8S1179 heterozygous sample containing 11 and 13 TATC repeats. In the bottom panel, 23 nt were removed compared with the top panel, which corresponds to a mass reduction of almost 8,000 Da. The peaks are sharper in the lower panel, as the products are blunt ended. Identical genotypes were obtained with both approaches, illustrating that the ddG termination is occurring at the same point on the two different sized alleles.

To summarize, STR sample sizes were reduced using primers that have been designed to bind close to the repeat region or even partially on the repeat itself. A cleavable primer was incorporated into the PCR product to allow post-PCR chemical cleavage and subsequent mass reduction. Two additional post-PCR methods were also explored to further reduce the measured DNA size. These methods

Exhibit 37. **Mass spectra of a D8S1179 sample illustrating the benefit of a dideoxynucleotide termination approach.** The top panel displays a result from a regular PCR product; the bottom panel contains the same sample treated with a linear amplification mix containing a ddG terminator with dA, dT, and dC deoxynucleotides. With the ddG approach, the problem of incomplete adenylation (both –A and +A forms of a PCR product) is eliminated, and the amplicons are smaller, which improves their sensitivity and resolution in the mass spectrometer. The red oval highlights the broader -A/+A peaks present in the regular PCR product. Note: The genotype (i.e., 11 and 13 repeats) is identical between the two approaches, even though almost 8,000 Da are removed with the ddG termination.



included restriction enzyme digestion in the flanking region on the other side of the repeat region from the cleavable primer and a primer extension through the repeat region with a single dideoxynucleotide terminator (single base sequencing approach).

To illustrate the advantages of these approaches to reduce the overall DNA product mass, researchers examined the STR locus TPOX. Using a conventional primer set, a sample containing 11 repeats measured 232 bp or ~66,000 Da. By redesigning the primers to anneal close to the repeat region, a PCR product of 89 bp was obtained. With the cleavable primer, the size was reduced to 69 nt or 21,351 Da. By incorporating a ddC termination reaction, another 20 nt were removed leaving only 49 nt or ~12,000 Da (primarily only the repeat region). The repeat region contained 44 nt (4 nt x 11 repeats) or ~10,500 Da. The ddC termination was also used in multiplex STR analysis to produce a CSF1PO-TPOX-TH01 triplex (exhibits 4 and 5). The repeat sequences used for these STR loci were AGAT for CSF1PO, AATG for TPOX, and AATG for TH01. The level of sequence clipping by ddC was as follows: CSF1PO (-14 nt), TPOX (-20 nt), and TH01 (-4 nt).

Multiplex STR Work

Due to the limited size range of DNA molecules that may be analyzed by this technique, a new approach to multiplexing was developed that involved interleaving alleles from different loci rather than producing nonoverlapping multiplexes. If the amplicons could be kept under ~25,000 Da, a high degree of mass accuracy and resolution could be used to distinguish alleles from multiple loci that may differ by only a fraction of a single nucleotide (exhibit 10). Allelic ladders are useful to demonstrate that all alleles in a multiplex are distinguishable (exhibit 9).

The expected masses for a triplex involving the STR loci CSF1PO, TPOX, and TH01 (commonly referred to as a CTT multiplex) are schematically displayed in exhibit 4. All known alleles for these STR loci, as defined by STRBase (Ruitberg et al., 2001), are fully resolvable and far enough apart to be accurately determined. For example, TH01 alleles 9.3 and 10 fall between CSF1PO alleles 10 and 11. For all three STR systems in this CTT multiplex, the AATG repeat strand is measured, which means that the alleles *within* the same STR system differ by 1,260 Da. The smallest spread between alleles *across* multiple STR systems in this particular multiplex exists between the TPOX and TH01 alleles, where the expected mass difference is 285 Da. TPOX and CSF1PO alleles differ by 314 Da, while TH01 and CSF1PO alleles differ by 599 Da. By using the same repeat strand in the multiplex, the allele masses between STR systems all stay the same distance apart. Each STR has a unique flanking region and it is these sequence differences between STR systems that permit multiplexing in such a fashion as described here. An actual result with this CTT multiplex is shown in exhibit 5. This particular sample is homozygous for both TPOX (8,8) and CSF1PO (12,12) and heterozygous at the TH01 locus (6,9.3).

It is also worth noting that this particular CTT multiplex was designed to account for possible, unexpected microvariants. For example, a CSF1PO allele 10.3 that appears to be a single base shorter than CSF1PO allele 11 was recently reported (Lazaruk et al., 1998). With the CTT multiplex primer set described here, a CSF1PO 10.3 allele would have an expected mass of 21,402 Da, which would be fully distinguishable from the nearest possible allele (i.e., TH01 allele 10) because these alleles would be 286 Da apart. Using a mass window of 100 Da as defined by previous precision studies (Butler et al., 1998), all possible alleles including microvariants should be fully distinguishable. STR multiplexes are designed so that expected allele masses between STR systems are offset in a manner that possible microvariants, which are most commonly insertions or deletions of a partial repeat unit, may be distinguished from all other possible alleles. The larger the allele's mass range, the more difficult it becomes to maintain a high degree of mass accuracy. For example, exhibit 8 shows the observed mass for TH01 allele 9.3 is -52 Da from its expected mass, while TPOX allele 9 is only 3 Da from its expected mass. In this particular case, the mass calibrants used were 4,507 Da and 10,998 Da. Thus, the TPOX allele's mass measurement was more accurate and closer to the calibration standard. The ability to design multiplexes that have a relatively compact mass range is important to maintaining the high level of mass accuracy needed for closely spaced alleles from different, overlapping STR loci. The mass calibration standards should also span the entire region of expected measurement to guarantee the highest degree of mass accuracy.

Two possible multiplexing strategies for STR genotyping are illustrated in exhibit 38. Starting with a single punch of blood stained FTA paper, it is possible to perform a multiplex PCR (simultaneously amplifying all STRs of interest) followed by another PCR with primer sets that are closer to the repeat region. With this approach, single or multiplexed STR products can be produced that are small enough for mass spectrometry analysis. Alternatively, multiple punches could be made from a single bloodstain on the FTA paper followed by singleplex or multiplex PCR with mass spectrometry primers. After the genotype is determined for each STR locus in a sample, the information would be combined to form a single sample genotype for inclusion in CODIS or some other DNA database. This multiplexing approach permits flexibility for adding new STR loci or only processing a few STR markers across a large number of samples at a lower cost than processing extensive and inflexible STR multiplexes.

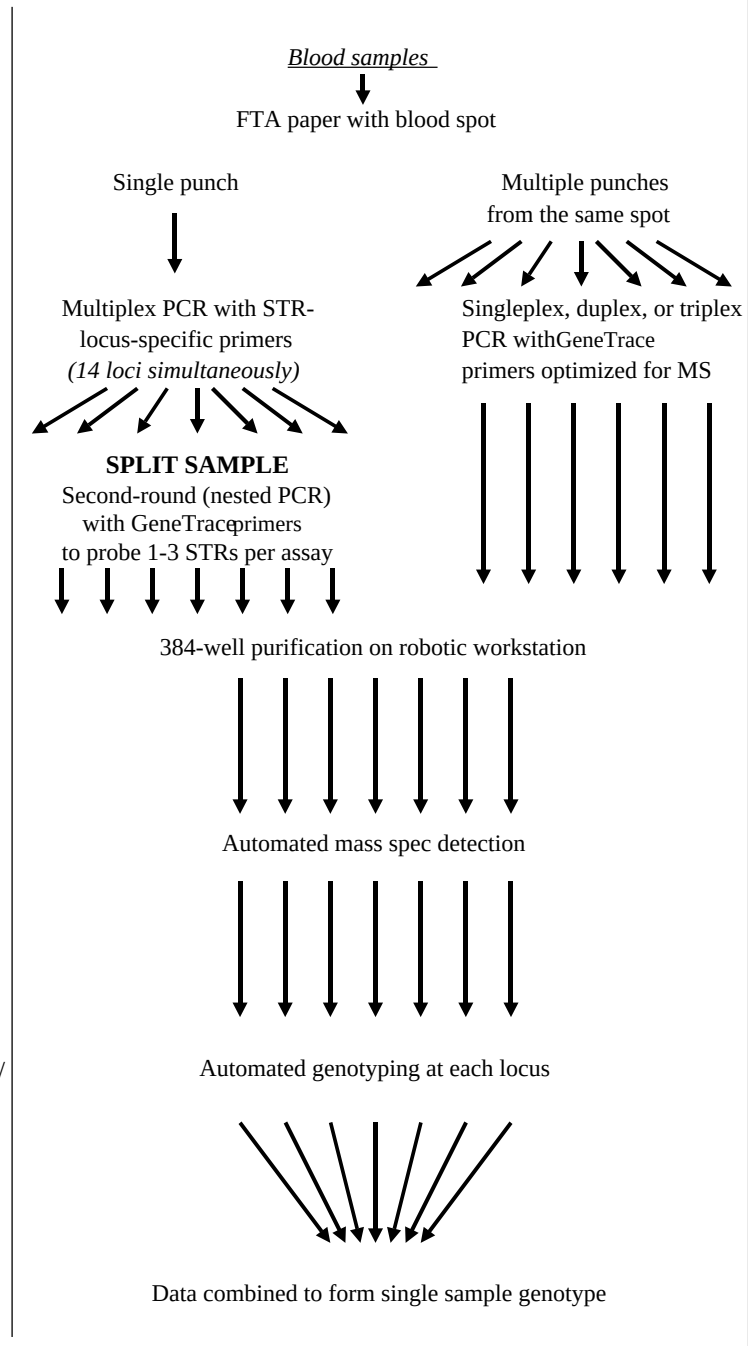
Comparison Tests Between ABI 310 and Mass Spectrometry Results

A plate of 88 samples from the CDOJ DNA Laboratory was tested with 10 different STR markers and compared with results obtained using the ABI 310 Genetic Analyzer and commercially available STR kits. The samples were supplied as a 200 µL aliquot of extracted genomic DNA in a 96-well tray with each sample at a concentration of 1 ng/µL. A 5 µL aliquot was used for each PCR reaction, or 5 ng total per reaction. Since each marker was amplified and examined individually, approximately 35 ng of extracted genomic DNA was required to obtain genotypes on the same 7 markers as were amplified in a single AmpF1STR® COfiler™ STR multiplex. Only 2 ng of genomic DNA were used per reaction with the AmpF1STR® COfiler™ kit. Thus, a multiplex PCR reaction is much better suited for situations where the quantity of DNA is limited (e.g., crime scene sample). However, in most cases

involving high-throughput DNA typing (e.g., offender database work), hundreds of nanograms of extracted DNA would be easily available.

A major advantage of the mass spectrometry approach is speed of the technique and the high-throughput capabilities when combined with robotic sample preparation. The data collection times required for the 88 CDOJ samples using the ABI 310 Genetic Analyzer and GeneTrace's mass spectrometry method are compared in exhibit 11. While it took the ABI 310 almost 3 days to collect the data for the 88 samples, the same genotypes were obtained on the mass spectrometer in less than 2 hours. Even the ability to analyze multiple STR loci simultaneously with different fluorescent tags on the ABI 310 could not match the speed of GeneTrace's mass spectrometry data collection with each marker run individually.

Exhibit 38. **Multiplexing strategies for STR genotyping using FTA™ paper**



To verify that the mass spectrometry approach produces accurate results, comparison studies were performed on the genotypes obtained from the two different methods across 8 different STR loci. Exhibits 12–19 contain a direct comparison with 1,408 possible data points (2 methods × 88 samples × 8 loci). With a few minor exceptions, there was almost a 100% correlation between the two methods. In addition to the data obtained on the 8 loci from both the ABI 310 and the mass spectrometer, two additional markers (D8S1179 and DYS391) were measured by mass spectrometry across these same 88 samples (exhibits 39–40). Both the D8S1179 and the DYS391 primer sets worked extremely well in the mass spectrometer (exhibit 41). Thus, it is likely that if results were made available on these same samples with fluorescent STR primer sets (e.g., D8S1179 is in the AmpF1STR® Profiler Plus™ kit), there would also be a further correlation between the two methods.

PCR Issues

Null alleles

When making comparisons between two methods that use different PCR primer sets, the issue is whether or not a different primer set for a given STR locus will result in different allele calls through possible sequence polymorphisms in the primer binding sites. In other words, do primers used for mass spectrometry that are closer to the repeat region than those primers used

Exhibit 39. **CDOJ D8S1179 STR results with the mass spectrometry method**

Position	Mass Spec	Allele 1 (Da)	Allele 2 (Da)	Position	Mass Spec	Allele 1 (Da)	Allele 2 (Da)
A1	12,15	24,693	28,228	A7	13,13	25,791	
B1	14,15	27,159	28,369	B7	13,14	25,845	26,954
C1	11,14	23,342	26,933	C7	12,15	24,540	28,101
D1	15,15	28,199		D7	13,14	25,772	26,931
E1	11,14	23,421	26,988	E7	14,14	26,960	
F1	13,17	25,822	30,529	F7	13,16	25,785	29,381
G1	11,14	23,449	27,093	G7	14,14	27,097	
H1	14,14	27,067		H7	14,14	27,039	
A2	13,13	25,777		A8	13,15	25,770	28,204
B2	12,14	24,597	27,007	B8	11,13	23,383	25,816
C2	11,11	23,361		C8	14,15	27,033	28,043
D2	11,13	23,399	25,768	D8	14,14	27,076	
E2	13,14	25,904	27,012	E8	14,15	26,984	28,221
F2	14,14	27,125		F8	13,14	25,781	26,946
G2	No data			G8	10,12	22,183	24,581
H2	11,15	23,344	28,151	H8	12,15	24,587	28,197
A3	15,15	28,160		A9	15,16	28,265	29,390
B3	13,15	25,904	28,256	B9	13,14	25,835	26,963
C3	12,14	24,640	27,022	C9	11,14	23,447	27,084
D3	11,15	23,407	28,201	D9	11,14	23,391	26,988
E3	11,14	23,461	27,093	E9	11,14	23,361	26,931
F3	14,15	27,086	28,186	F9	14,15	27,031	28,225
G3	10,15	22,218	28,230	G9	14,14	26,978	
H3	13,16	25,849	29,355	H9	11,13	23,451	26,008
A4	14,15	27,018	28,112	A10	13,14	25,789	26,841
B4	12,14	24,605	27,018	B10	15,15	28,206	
C4	13,13	25,797		C10	13,13	25,791	
D4	No data			D10	14,15	26,999	28,123
E4	14,14	27,108		E10	13,14	25,895	26,980
F4	14,15	27,080	28,215	F10	13,15	25,777	28,197
G4	12,13	24,311	25,519	G10	14,15	26,733	27,909
H4	13,14	25,824	27,044	H10	12,14	24,688	27,093
A5	12,16	24,548	29,321	A11	12,15	24,574	28,230
B5	13,14	25,893	26,997	B11	13,14	25,822	27,054
C5	12,13	24,727	25,885	C11	14,16	26,963	29,324
D5	No data			D11	13,14	25,812	26,950
E5	14,14	26,960		E11	12,15	24,617	28,232
F5	13,14	25,916	27,014	F11	14,14	26,982	
G5	14,15	27,009	28,106	G11	14,14	27,005	
H5	13,14	25,804	26,708	H11	12,13	24,631	25,818
A6	14,14	26,988					
B6	14,16	26,982	29,364				
C6	15,15	28,249					
D6	14,14	26,716					
E6	13,16	25,933	29,584				
F6	8,14	19,884	27,041				
G6	15,15	28,394					
H6	14,14	27,009					

in fluorescent STR typing yield the same genotype?

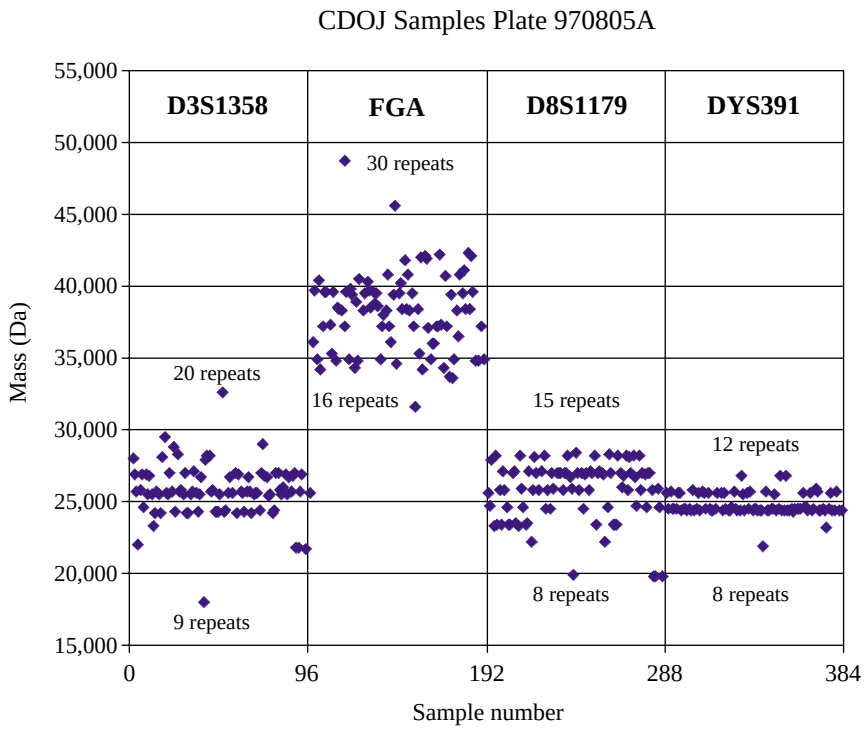
Differences between primer sets are possible if there are sequence differences outside the repeat region that occur in the primer binding region of either set of primers (exhibit 42). This phenomenon produces what is known as a "null" allele, or in other words, the DNA template exists for a particular allele but fails to amplify during PCR due to primer hybridization problems. In all cases except the STR locus D7S820, there was excellent correlation in genotype calls between the two methods (where mass spectrometry and CE results were obtained), signifying that the mass spectrometry primers did not produce any null alleles.

For the STR locus D7S820, 17 of 88 samples did not agree with the two methods (exhibit 18). The bottom two panels in exhibit 43 illustrate more microheterogeneity at this locus than previously reported. On the lower left plot, only the allele 10 peak can be seen; allele 8, which was seen with PCR amplification using a fluorescent primer set, is missing (see position of red arrow in exhibit 43). On the lower right plot, both allele 8 and allele 10 are amplified and detected in the mass spectrometer, confirming that the problem is with the PCR amplification and not the mass spectrometry data collection. In this particular case, there is a difference between those two alleles 8, meaning that the mass spectrometer primer set identified a new, previously unreported allele. When using fluorescent primer sets that anneal 50–100 bases or more from the repeat region, a single-base change (e.g., T to C) out of a 300 bp PCR product is difficult to detect. Upon comparing the results of mass spectrometer data where there were missing alleles with the results from the ABI 310, it was noted that the situation occurred only with some allele 8s, 9s, and 10s (see underlined alleles in the ABI 310 column of exhibit 18). Thus, these null alleles were variants of alleles with 8, 9, or

Exhibit 40. **CDOJ DYS391 STR results with the mass spectrometry method**

Position	Mass Spec	Allele 1 (Da)	Position	Mass Spec	Allele 1 (Da)
A1	10	24,489	A7	10	24,416
B1	11	25,672	B7	10	24,406
C1	10	24,487	C7	10	24,353
D1	10	24,455	D7	8	21,905
E1	10	24,471	E7	11	25,662
F1	11	25,641	F7	10	24,353
G1	11	25,637	G7	10	24,422
H1	10	24,436	H7	10	24,451
A2	10	24,459	A8	10	24,455
B2	10	24,465	B8	11	25,529
C2	10	24,440	C8	10	24,410
D2	10	24,455	D8	10	24,473
E2	10	24,359	E8	12	26,841
F2	11	25,837	F8	10	24,438
G2	10	24,444	G8	10	24,359
H2	10	24,451	H8	12	26,805
A3	11	25,639	A9	10	24,367
B3	10	24,414	B9	10	24,416
C3	11	25,654	C9	10	24,463
D3	11	25,591	D9	10	24,343
E3	10	24,463	E9	10	24,471
F3	11	25,648	F9	10	24,457
G3	10	24,475	G9	10	24,465
H3	10	24,436	H9	10	24,479
A4	10	24,408	A10	11	25,631
B4	10	24,475	B10	10	24,617
C4	11	25,625	C10	10	24,560
D4	No data		D10	10	24,444
E4	11	25,581	E10	11	25,650
F4	10	24,396	F10	10	24,414
G4	11	25,562	G10	10	24,453
H4	10	24,463	H10	11	25,866
A5	10	24,390	A11	11	25,662
B5	10	24,446	B11	10	24,432
C5	10	24,599	C11	10	24,400
D5	11	25,652	D11	10	24,463
E5	10	24,451	E11	10	24,420
F5	10	24,436	F11	9	23,175
G5	10	24,380	G11	10	24,459
H5	12	26,775	H11	11	25,585
A6	11	25,550			
B6	10	24,428			
C6	11	25,583			
D6	10	24,457			
E6	11	25,658			
F6	10	24,436			
G6	10	24,473			
H6	10	24,463			

Exhibit 41. **Plot of measured masses versus sample number from four different STR loci.** FGA, which has a high degree of scatter, is the most polymorphic marker and has the largest mass alleles. The highest and lowest alleles observed for each STR locus in this study are shown on the plot.



10 repeats. Most likely, a sequence microvariant occurs within the repeat region near the 3'-end of the reverse primer, which anneals to two full repeats. Unfortunately, time constraints restricted the gathering of sequence information for these samples to confirm the observed variation. Interestingly enough, the D7S820 locus has been reported to cause similar null allele problems with other primer sets (Schumm et al., 1997).

Microvariants

Sequence variation between alleles can take the form of insertions, deletions, or nucleotide changes. Alleles containing some form of sequence variation compared with more commonly observed alleles are often referred to as microvariants because they are slightly different from full repeat alleles. For example, the STR locus TH01 contains a 9.3 allele, which has 9 full repeats (AATG) and a partial repeat of 3 bases (ATG). In this particular example, the 9.3 allele differs from the 10 allele by a single base deletion of adenine. Microvariants exist for most STR loci and are being identified in greater numbers as more samples are being examined around the world. In this study, three previously unreported STR microvariants (exhibit 44) were discovered during the analysis of 38 genomic DNA samples from a male population data set provided by Dr. Oefner (exhibit 45). These microvariants occurred in the three most polymorphic STR loci that possess the largest and most complex repeat structures: FGA, D21S11, and D18S51.

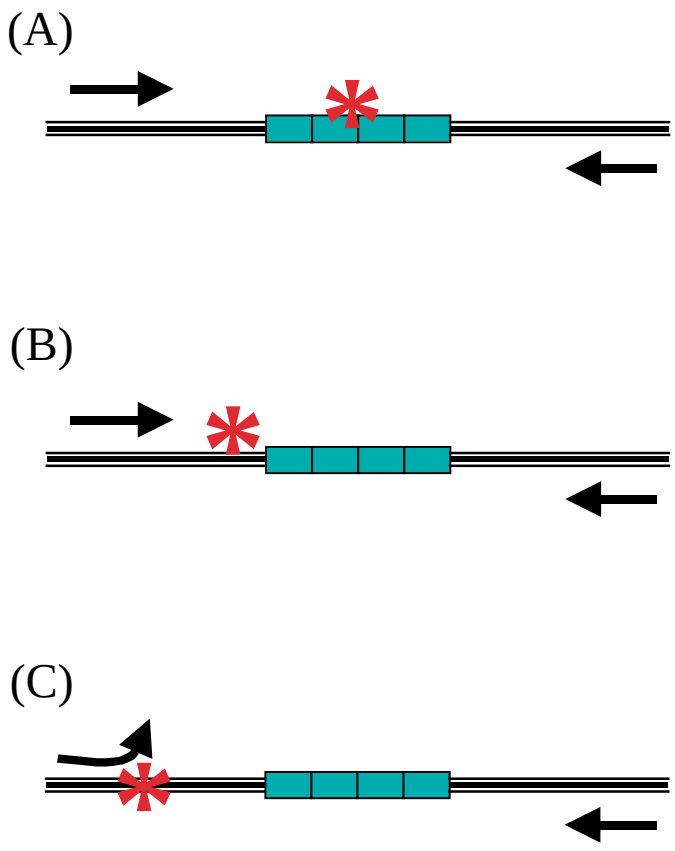
The ability to make accurate mass measurements with mass spectrometry is a potential advantage when locating new microvariants. If the mass precision is good, then any peaks that have large offsets from the expected full repeat alleles could be suspect microvariants in the form of insertions or deletions because their masses would fall outside the expected variance due to instrument variation. This possibility is especially true when working with heterozygous samples. Microvariants can be detected by using the mass difference between the two alleles and comparing this value with the expected value for full repeats or with the allele peak mass offsets. If the peak mass offsets shift together, then both alleles are full repeats, but if one of the peak mass offsets is significantly different (e.g., ~300 Da), a possible insertion or deletion exists in one of the alleles. Exhibit 46 illustrates this concept by plotting the mass offset (from a calculated allele mass) of allele 1 verses the mass offset (from a calculated allele mass) of allele 2. Note that the 9.3 microvariant (i.e., partial) repeat alleles for TH01 cluster away from the comparison of full repeat versus full repeat allele. On the other hand, results for the other three STR loci, which have no known microvariants in this data set, have mass offsets that shift together for the heterozygous alleles. Exhibit 47 compares the peak mass offsets for the amelogenin X allele with the Y allele and demonstrates that full "repeats" shift together during mass spectrometry measurements.

Nontemplate addition

DNA polymerases, particularly the *Taq* polymerase used in PCR, often add an extra nucleotide to the 3'-end of a PCR product as template strands are copied. This nontemplate addition—which is most often an adenine, hence, the term "adenylation"—can be favored by adding a final incubation step at 60 °C or 72 °C after the

temperature cycling steps in PCR (Clark, 1988, and Kimpton et al., 1993). However, the degree of adenylation is dependent on the sequence of the template strand, which in the case of PCR results from the 5'-end of the reverse primer. Thus, every locus will have different adenylation properties because the primer sequences are different. From a measurement standpoint, it is better to have all molecules of a PCR product as similar as possible for a particular allele. Partial adenylation, where some of the PCR products do not have the extra adenine (i.e., -A peaks) and some do (i.e., +A peaks), can contribute to peak broadness if the separation system's resolution is poor (see top panel of exhibit 37). Sharper peaks improve the likelihood that a system's genotyping software can make accurate calls. Variation in the adenylation status of an allele across multiple samples can have an impact on accurate sizing and genotyping potential microvariants. For example, a nonadenylated TH01 10 allele would look the same as a fully adenylated TH01 9.3 allele in the mass spectrometer because their masses are identical. Therefore, it is beneficial if all PCR products for a particular amplification are either +A or -A rather than a mixture (e.g., ±A). By using the temperature soak at the end of thermal cycling, most of the STR loci were fully adenylated, with the notable exception of TPOX, which was typically nonadenylated, and TH01, which under some PCR conditions produced partially adenylated amplicons. For making correct genotype calls, the STR mass ladder file (exhibit 30) was altered according to the empirically determined adenylation status.

Exhibit 42. **Effects of sequence variation on PCR amplification in or around STR repeat regions.** The asterisk symbolizes a DNA difference (base change, insertion, or deletion of a nucleotide) from a typical allele for a STR locus. In situation (A), the variation occurs within the repeat region (depicted in green) and should have no impact on the primer binding and the subsequent PCR amplification, although the overall amplicon size may vary slightly. In situation (B), the sequence variation occurs just outside the repeat in the flanking region but interior to the primer annealing sites. Again, PCR should not be affected, although the size of the PCR product may vary slightly. However, in situation (C), the PCR can fail due to a disruption in annealing a primer because the primer no longer perfectly matches the DNA template sequence. Therefore, if sequence variation occurs in the flanking region for a particular locus, one set of primers may work while another may fail to amplify the template. The template would therefore be a "null" allele.



During the course of this project, Platinum® GenoTYPE™ *Tsp* DNA polymerase (Life Technologies, Rockville, MD) became available that exhibits little to no nontemplate nucleotide addition. This new DNA polymerase was tested with STR loci that had been shown to produce partial adenylation to see if the +A peak could be eliminated. Exhibit 48 compares mass spectrometry results obtained using AmpliTaq Gold (commonly used) polymerase with the new *Tsp* polymerase. The *Tsp* polymerase produced amplicons with only the -A peaks, while TaqGold showed partial adenylation with these TH01 primers. Thus, this new polymerase has the potential to produce sharper peaks (i.e., no partial adenylation) and allele masses that can be more easily predicted

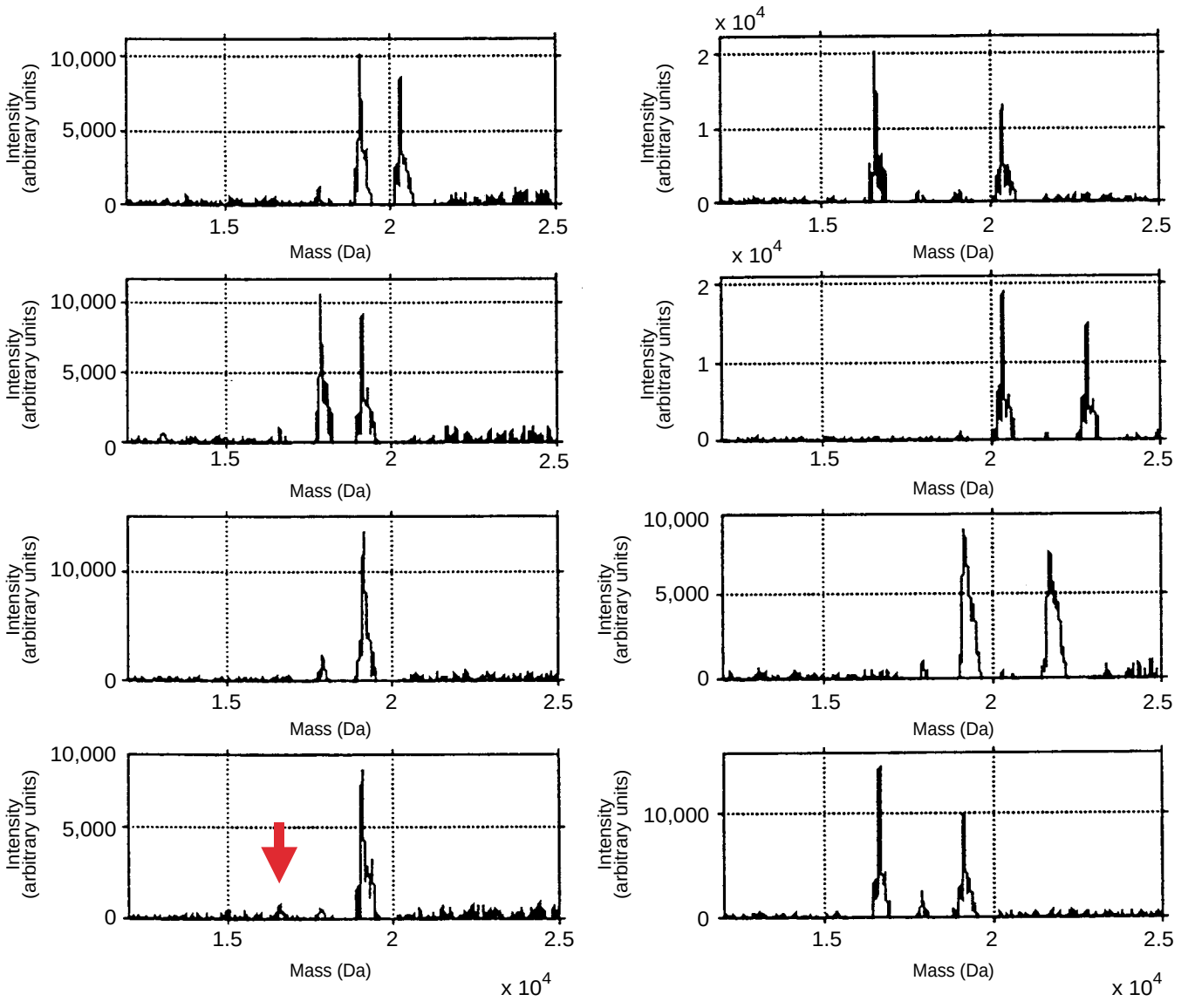
(i.e., all PCR products would be nonadenylated).

Stutter products

During PCR amplification of STR loci, repeat slippage can occur and result in the loss of a repeat unit as DNA strand synthesis occurs through a repeated sequence. These stutter products are typically 4 bases, or one tetranucleotide repeat, shorter than the true allele PCR product. The amount of stutter product compared to the allele product varies depending on the STR locus and the length of the repeat, but typically stutter peaks are 2–10% of the allele peak height (Walsh et al., 1996). Forensic DNA scientists are concerned about stutter products because their presence can interfere in the interpretation of DNA mixture profiles.

When reviewing plots of GeneTrace's mass spectrometry results for STR loci, forensic scientists have commented on the reduced level of stutter product

Exhibit 43. **Mass spectra of CDOJ samples amplified with D7S820 primers.** From left side, top-to-bottom followed by right side, top-to-bottom, sample genotypes are (10,11), (9,10), (10,10), (null allele 8, 10), (8,11), (11,13), (10,12), and (8,10). The arrow indicates the position where allele 8 should be present in the sample but is missing due to a primer annealing binding site sequence polymorphism that results in a "null allele." The mass range shown here is 12,000–25,000 Da.



detection (exhibit 35). There are two possibilities for this reduction:

- Since the primers are closer to the repeat region, smaller PCR products are amplified, which means that the DNA polymerase does not have to hold on to the extending strand as long for synthesis purposes. It is possible that the polymerase reads through the repeat region "faster" and, therefore, the template strands do not have as much of an opportunity to slip and reanneal out of register on the repeat region. For example, *Taq* polymerase has a processivity rate of ~60 bases before it falls off the extending DNA strand; therefore, the closer the PCR product size is to 60 bases, the better the extension portion of the PCR cycle. GeneTrace's PCR product sizes, which are typically less than 100 bp, are much smaller than the

Exhibit 44. **Electropherograms of ABI 310 results for new STR microvariants seen in the Stanford male population samples.** The D18S51 16.2 allele, D21S11 30.3 allele, and FGA 28.1 allele have not been reported previously in the literature. These plots are views from Genotyper 2.0 with results overlaid on shaded allele bins. The base pair size range is indicated at the top of each plot. Note: The microvariant alleles (indicated by the red arrows) fall between the shaded bins, but the other alleles in the heterozygote set contain complete repeats and fall directly on the shaded (expected) allele bin.

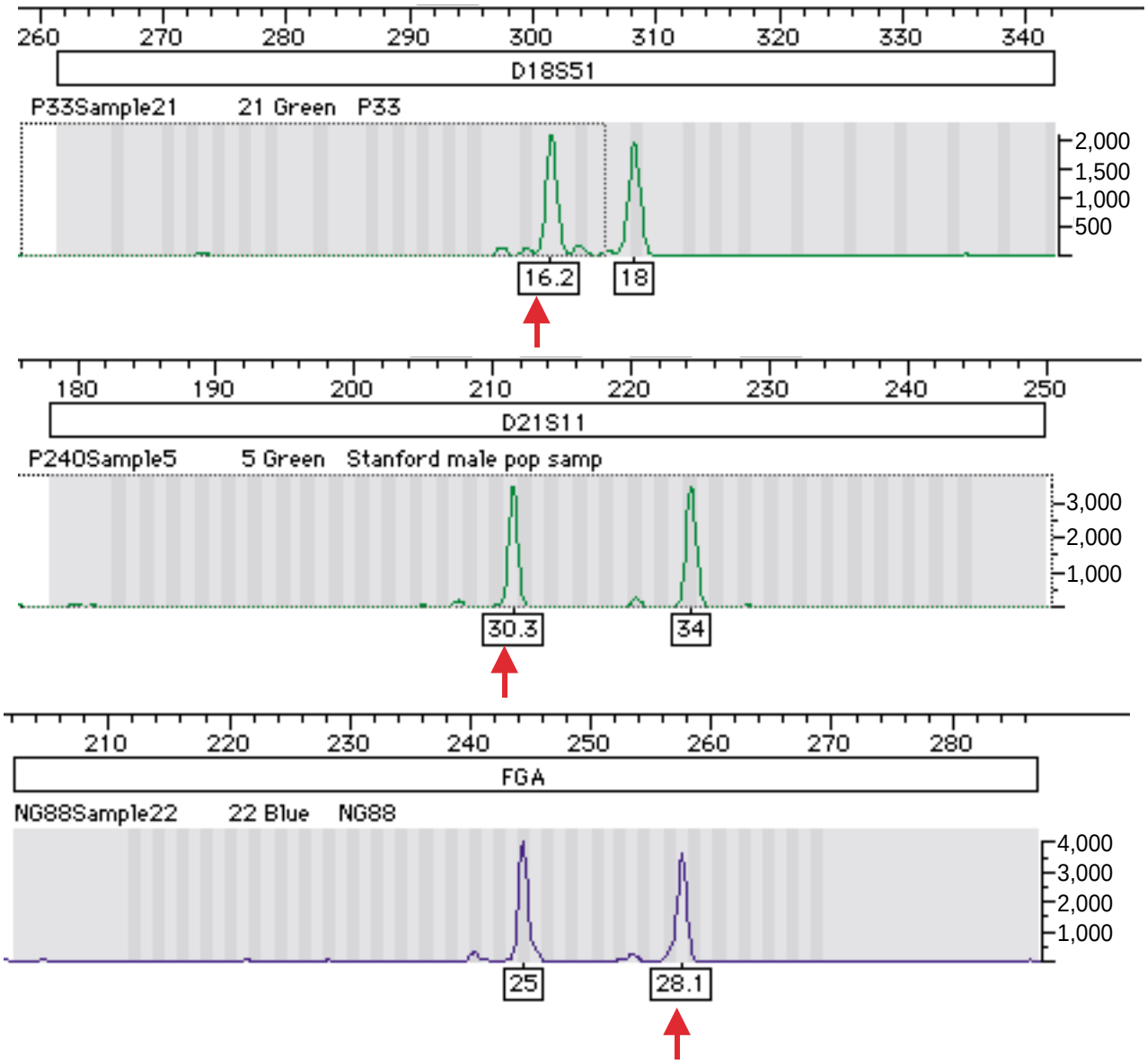


Exhibit 45. **ProfilerPlus™ results from Stanford male population samples**

Sample Name	Amel-A1	Amel-A2	D8-A1	D8-A2	D21-A1	D21-A2	D18-A1	D18-A2	D3-A1	D3-A2	VWA-A1	VWA-A2	FGA-A1	FGA-A2	D5-A1	D5-A2	D13-A1	D13-A2	D7-A1	D7-A2
Aus21	X	Y	8	12	29	31.2	12	18	15	17	14	17	18	22	12	12	11	12	9	11
Aus28	X	Y	14	15	29	31.2	15	19	16	17	15	17	19	20	12	12	10	12	8	10
Berg15	X	Y	10	12,13	28,30.2	31.2	12,17	14	15,17	18,19	16	17,18	20,21	24	11	13w	8,14	11	7w	10
Berg19	X	Y	14	14	32.2	32.2	12	18	16	16	14	18	22	22	12	13	12	12	9	13
BI12	X	Y	12	12	30	31.2	17	17	16	18	17	19	24	27	12	12	11	11	9	11
Bsk092	X	Y	14	14	29	33.2	13	16	17	17	17	17	23	23	11	12	12	12	11	11
Bsk111	X	Y	12	13	30	32.2	16	19	14	14	16	16	18	19	12	12	8	10	8	9
Bsk118	X	Y	13	14	30.2	30.2	11	14	16	16	17	17	23	25	10	12	8	10	8	8
CH17	X	Y	12	14	32.2	34.2	14	23	15	15	16	18	20	26	9	12	8	12	11	12
CH23	X	Y	12	13	29	31	16	16	15	15	14	17	24	24	11	12	9	12	10	12
CH42	X	Y	12	14	28.2	31.2	13	15	16	16	17	17	21	22	12	12	10	12	11	11
F18	X	Y	14	14	31	32.2	19	19	15	15	14	16	21	23	13	13	11	12	8	12
F21	X	Y	14	15	27	31	14	19	15	15	17	18	22	26	12	13	11	13	8	8
J13	X	Y	14	14	32.2	33.2	17	19	15	17	14	16	20	22	10	11	8	11	10	10
J3	X	Y	13	14	30	30	13	15	16	16	17	17	22	24	10	14	8	11	10	10
J37	X	Y	10	13	31	31.2	13	20	15	15	16	18	22	25	10	12	8	11	10	10
J39	X	Y	13	14	30	31.2	13	13	15	18	17	17	23	24	11	13	12	13	8	12
JK2921	X	Y	10	10	29	31.2	14	15	15	16	14	19w	19	22	10	11	11	11	9	9
JK2979	X	Y	10	12	32.2	33.2	14	14	15	16	14	18	21	24.2	11	14	11	12	11	11
MDK204	X	Y	14	17	28	30.2	12	19	14	15	16	16	23	25	12	13	11	12	8	10
Mel12	X	Y	13	14	30	32.2	17	20	15	16	17	18	21	22	13	14,15	11	12	12	12
Mel15	X	Y	14	15	28	31.2	14,15	22	16	18	16	18	23	24	10	10	12	13	11	11
Mel18	X	Y	15	15	28	32.2	14	15	15	16	17	18	24	24	10	11	12	13	10	11
GN 83	X	Y	13	13	30	31	1 3	18	15	16	1 5	1 7	23	24	11	11	8	8	8	10
NG85	X	Y	15	15	29	31.2	15	21	16	17	17	19	23	26	13	13	8	8	11	12
NG88	X	Y	12	15	33.2	38.2	13	17	15	15	16	16	25	28.1	11	11	8	11	8	10
OM135	X	Y	12	13	28	39	15	16	16	16	16	18	21	25	12	12	12	12	11	11
P103G	**X**	**X**	12	17	29	30	19	20	17	17	15	19	20	24	13	13	10	12	8	8
P109	X	Y	11	14	28	34	17	21	15	16	15	19	22	27	11	12	12	12	9	10
P205	X	Y	11	15	28	36	15	15	14	15	15	16	21	24	8	10	12	12	9	10
P240	X	Y	14	14	30.3	34	16	16	16	17	15	21	22	22.2	10	11	8	13	11	12
P33	X	Y	13	14	29	30	16.2	18	14	17	16	17	20	24	8	10	11	12	10	10
P37G	X	Y	14	15	29	32.2	14	20	16	17	17	18	24	24	12	13	9	12	8	11
P37G?	X	Y	14	15	29	32.2	14	20	16	17	17	18	24	24	12	13	9	12	8	11
P73	X	Y	14	15	29	30	14	16	15	16	14	19	22.2	24	12	12	11	13	10	11
PG1162	X	Y	8	14	31.2	32.2	14	15	15	16	16	18	18	23	11	12	9	11	8	10
PKH062	X	Y	12	15	29	31	13	16	16	18	16	18	21	22	11	11	9	13	10	12
SDH053	X	Y	10	12	30	33.2	16	16	18	18	17	17	21	25	11	12	8	8	8	8

Note: The STR loci are color coded to indicate their fluorescent dye label color; the shaded boxes in the body of the exhibit refer to the microvariant alleles (exhibit 44), 3-banded patterns, or an unexpected "x,x" amelogenin.

Exhibit 46. **Plot of allele mass offsets (allele 1 versus allele 2) for heterozygous samples from four different loci.** These are 88 CDOJ samples for the STR loci TH01, TPOX, CSF1PO, and D16S539.

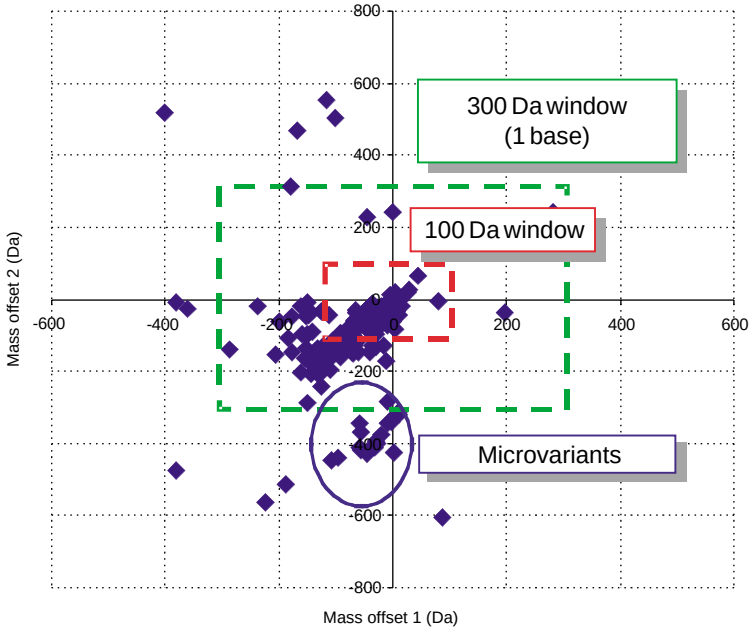
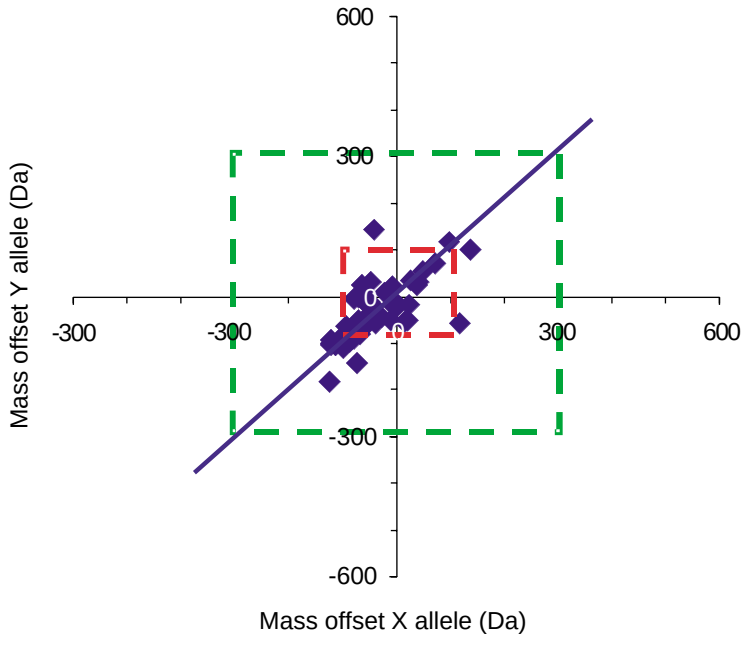


Exhibit 47. **Plot of X allele mass offset versus Y allele mass offset for 88 amelogenin samples.** The red box shows ±100 Da around the expected values and the green box shows ±300 Da. The blue line is the ideal situation where "heterozygous" peaks would shift in unison compared with the expected masses.



fluorescently labeled primer sets used by most forensic DNA laboratories (exhibit 2). However, this needs to be studied more extensively with multiple primer sets on a particular STR locus that generates various sized amplicons. For example, the primer sets described in exhibit 24 could be fluorescently labeled and analyzed on the ABI 310, where the stutter product peak heights could be quantitatively compared to the allele peak heights.

- The more likely reason that less stutter is observed by mass spectrometry is that the signal-to-noise ratio is much lower in mass spectrometry than in fluorescence measurements. Fluorescence techniques have a much lower background and are more sensitive for the detection of DNA than mass spectrometry. Thus, stutter may be present at similar ratios compared with those observed in fluorescence measurements, but because stutter is part of the baseline noise of mass spectrometry data, it may not be seen in the mass spectrum. This latter explanation is probably more likely, as indicated in very strong stutter peaks for some dinucleotide repeat markers (exhibit 49).

Whether stutter products are present or not, GeneTrace's current STR genotyping software has been designed to recognize them and not call them as alleles.

Primer sequence determinations from commercial STR kits

Primarily, two commercial manufacturers supply STR kits to the forensic DNA community: Promega Corporation and Applied Biosystems. These kits come with PCR primer sequences that permit simultaneous multiplex PCR amplification of up to 16 STR loci. One of the primers for each STR locus is labeled with a fluorescent

Exhibit 48. **Mass spectra comparing an STR sample amplified with TaqGold polymerase and Tsp polymerase.** The Tsp polymerase favors production of the nonadenylated form of PCR products, which results in a single peak for each allele (bottom panel). TaqGold produces a mixture of –A and +A peaks, which leads to two peaks for each allele (top panel). The peak masses in Daltons are indicated next to each peak. Mass difference measurements of 308 Da and 305 Da between the +A and –A peaks reveal that a "T" is added by TaqGold instead of the expected "A" (expected masses: T = 304 Da and A = 313 Da). The sample's genotype was TH01 6,8.

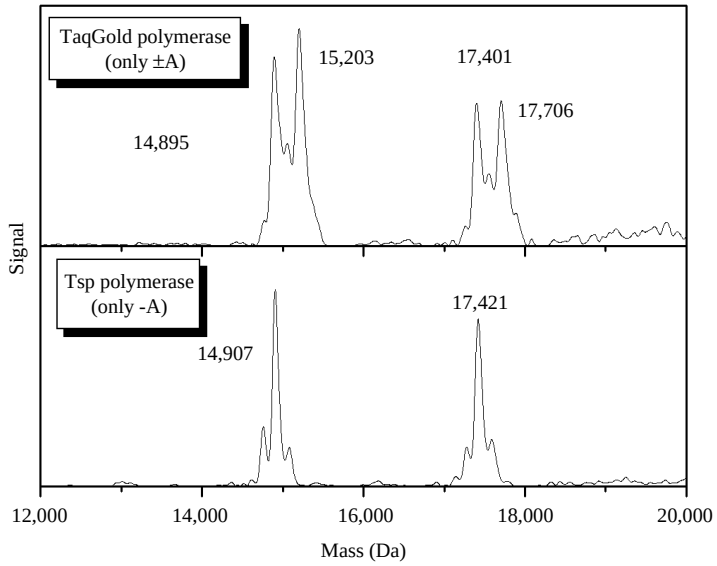
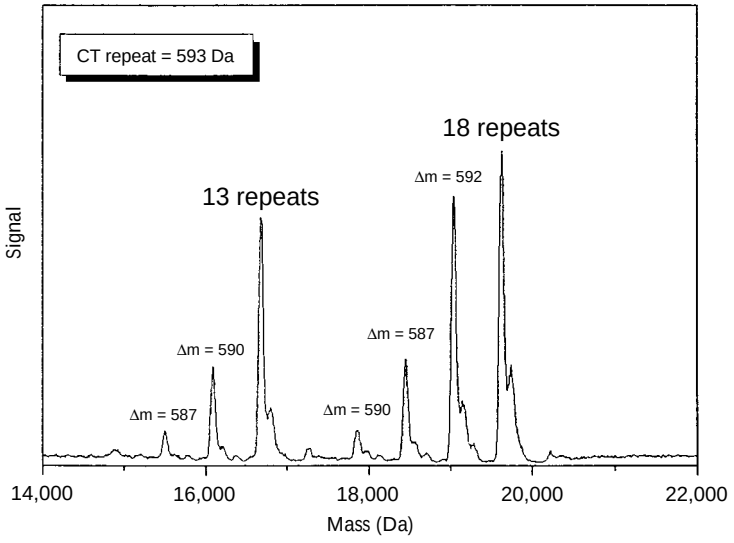


Exhibit 49. **Mass spectrum demonstrating detection of stutter products from a particularly stutter-prone dinucleotide repeat locus.** The mass differences between the stutter product peaks and the allele peaks can be used to determine the repeat sequence that is present on the measured DNA strand. Note: The amount of stutter is larger in the longer repeat allele than in the shorter allele.



dye to permit fluorescent detection of the labeled PCR products. Since the primer sequences are not disclosed by the manufacturers, mass spectrometry was used to determine where they annealed to the STR sequences compared with GeneTrace primers (see previous discussion on null alleles).

First, the primer mixtures were spotted and analyzed to determine each primer's mass (top panel of exhibit 50). Then a 5'→3' exonuclease was added to the primer mix and heated to 37 °C for several minutes to digest the primer one base at a time. An aliquot was removed every 5–10 minutes to obtain a time course on the digestion reaction. Each aliquot was spotted in 3-hydroxypicolinic acid matrix solution (Wu et al., 1993), allowed to dry, and analyzed in the mass spectrometer.

A digestion reaction produces a series of products that differ by one nucleotide. By measuring the mass difference between each peak, the original primer sequence may be determined (bottom panel of exhibit 50). Only the unlabeled primers will be digested because the covalently attached fluorescent dye blocks the 5'-end of the dye-labeled primer. Using only a few bases of sequence (e.g., 4–5 bases), it is possible to make a match on the appropriate STR sequence obtained from GenBank to determine the 5'-end of the primer without the fluorescent label.

With the full-length primer mass obtained from the first experiment, the remainder of the unlabeled primer can be identified. The position of the 5'-end of the other primer can be determined using the GenBank sequence and the PCR product length for the appropriate STR allele listed in GenBank (exhibit 2). The sequence of the labeled primer can be ascertained by using the appropriate primer mass determined from the first experiment and subtracting the mass of the fluorescent dye. The primer mass is then used to obtain the correct length of the primer on the GenBank sequence and the primer's

Exhibit 50. **Primer sequence determination with exonuclease digestion and mass difference measurements.** This example is a D5S818 primer pair purchased from Promega Corporation and used in its PowerPlex™ STR kit. The top panel shows a mass spectrum of the original primer pair prior to digestion. The bottom panel is the mass spectrum of the same primers following a 6 minute digestion at 37 °C with calf spleen phosphodiesterase, which is a 5'→3' exonuclease. The dye-labeled primer is not digested because the dye protects the 5'-end of the primer. Mass difference measurements between the digestion peaks leads to the sequence determination of the 5'-end of the unlabeled primer (see underlined portion of forward sequence). The determined sequences are 5'-GGTGATTTTCCTCTTTGGTATCC-3' (forward) and 5'-fluorescein dye—TTTACAACATTTGTATCTATATCTGT-3' (reverse).

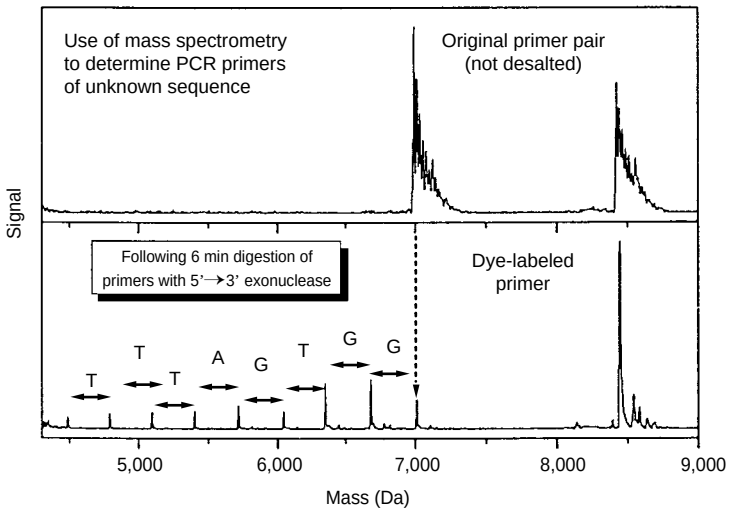
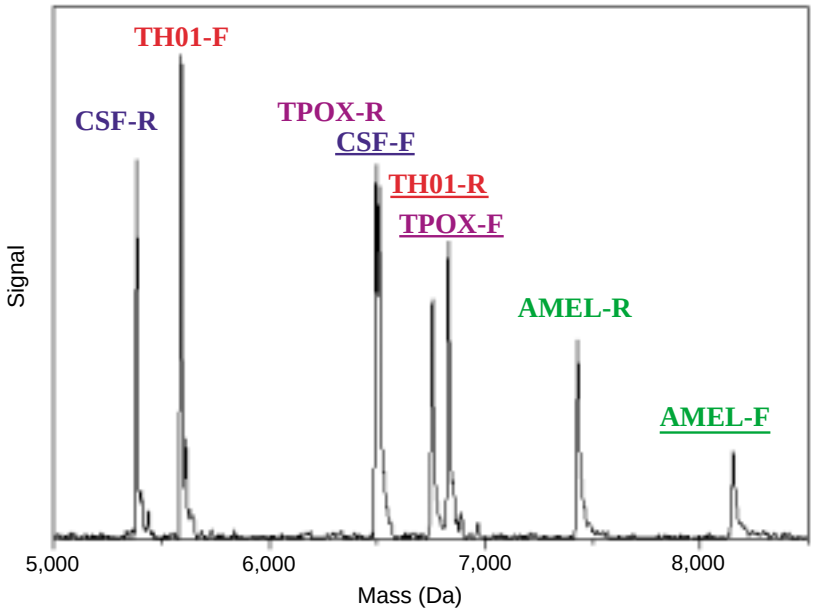


Exhibit 51. **Mass spectrum of AmpF1STR® Green I primer mix.** Each peak has been identified with its corresponding primer. Peaks containing the fluorescent dye (JOE) are underlined.



sequence. Finally, an entire STR multiplex primer set can be measured together in the mass spectrometer to observe the primer balance (exhibit 51). High-performance liquid chromatography fraction collection can be used to pull primers apart from complex, multiplex mixtures, and each primer can be identified as previously described. The primer sequences from both Promega and Applied Biosystems STR loci TH01 (exhibit 52), TPOX (exhibit 53), and CSF1PO (exhibit 54) were identified using this procedure. A comparison of the primer sequences from the two manufacturers found that they were very similar. The 3'-ends of the primer sets—the most critical portions for annealing during—were almost identical between the different kits. The ABI primers were typically shorter at the 5'-end and, therefore, produced PCR products that were ~10 bases shorter than those produced by the corresponding Promega primers. In all three STR loci, the primers annealed further away from the repeat region than the GeneTrace primer sets.

Analytical Capabilities of This Mass Spectrometry Method

Using the current primer design strategy, most STR alleles ranged in size from ~10,000 Da to ~40,000 Da. In mass spectrometry, the smaller the molecule, the easier it is to ionize and detect (all other things being equal). Resolution, sensitivity, and accuracy are usually better the smaller the DNA molecule being measured. Because the possible STR alleles are relatively far apart, reliable genotyping is readily attainable even with DNA molecules at the higher mass region of the spectrum. For example, neighboring full-length alleles for a tetranucleotide repeat, such as AATG, differ in mass by 1,260 Da.

Exhibit 52. **TH01 STR primer positions for commercially available primers highlighted on the GenBank sequence.** The forward primer is shown in blue with the reverse primer in brown. The repeat is highlighted in green on the strand that contains the fluorescent dye. The 3'-positions of the forward primers are identical but differ by a single base for the reverse primers. Promega primers are longer at the 5'-end, which produces a larger PCR product by 11 bp (6 bases on forward and 5 bases on reverse).

AmpFlSTR® Green I Kit

Reverse primer is labeled with JOE dye (fluorescein derivative)

PCR product =184 bp (9 repeats)

PowerPlex™ Kit

Forward primer is labeled with TMR dye (tetramethylrhodamine)

PCR product = 195 bp (9 repeats)

Exhibit 53. **TPOX STR primer positions for commercially available primers highlighted on the GenBank sequence.** The forward primer is shown in blue with the reverse primer in brown. The repeat is highlighted in green on the strand that contains the fluorescent dye. The 3'-positions of the reverse primers are identical but differ by a single base for the forward primers. Promega primers are longer at the 5'-end, which produces a larger PCR product by 7 bp (4 bases on forward and 3 bases on reverse).

AmpFlSTR® Green I Kit

Forward primer is labeled with JOE dye (fluorescein derivative)

PCR product = 237 bp (11 repeats)

PowerPlex™ Kit

Reverse primer is labeled with TMR dye (tetramethylrhodamine)

PCR product = 244 bp (11 repeats)

Exhibit 54. **CSF1PO STR primer positions for commercially available primers highlighted on the GenBank sequence.** The forward primer is shown in blue with the reverse primer in brown. The repeat is highlighted in green on the strand that contains the fluorescent dye. The 3'-positions of the reverse primers are identical but differ by a single base for the forward primers. Promega primers are longer at the 5'-end, which produces a larger PCR product by 11 bp (5 bases on forward and 6 bases on reverse).

AmpFlSTR® Green I Kit

Forward primer is labeled with JOE dye (fluorescein derivative)

PCR product = 304 bp (12 repeats)

PowerPlex™ Kit

Forward primer is labeled with TMR dye (tetramethylrhodamine)

PCR product = 315 bp (12 repeats)

Exhibit 55. **Mass spectrum of a TH01 allelic ladder reamplified from AmpF1STR® Green I allelic ladders.** The PCR product size of allele 10 is only 83 bp with a measured mass of 20,280 Da and separation time of 204 µs. The allele 9.3 and allele 10 peaks, which are only a single nucleotide apart, differ by only 1.5 µs on a separation timescale and can be fully resolved with this method.

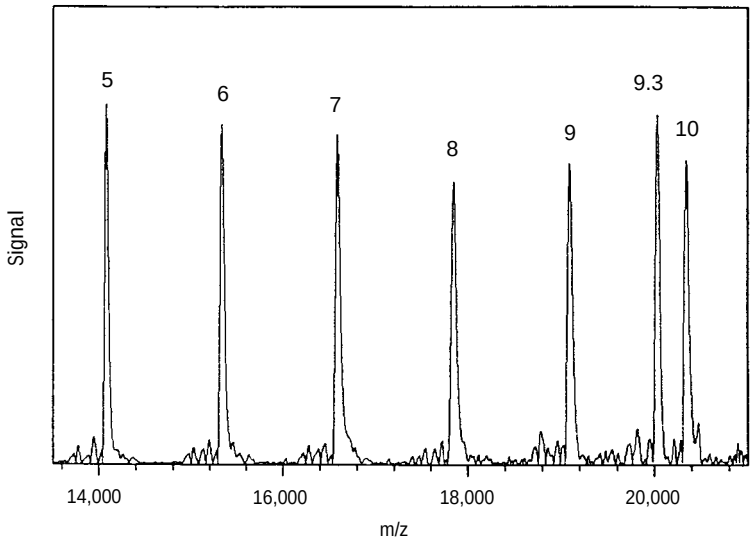
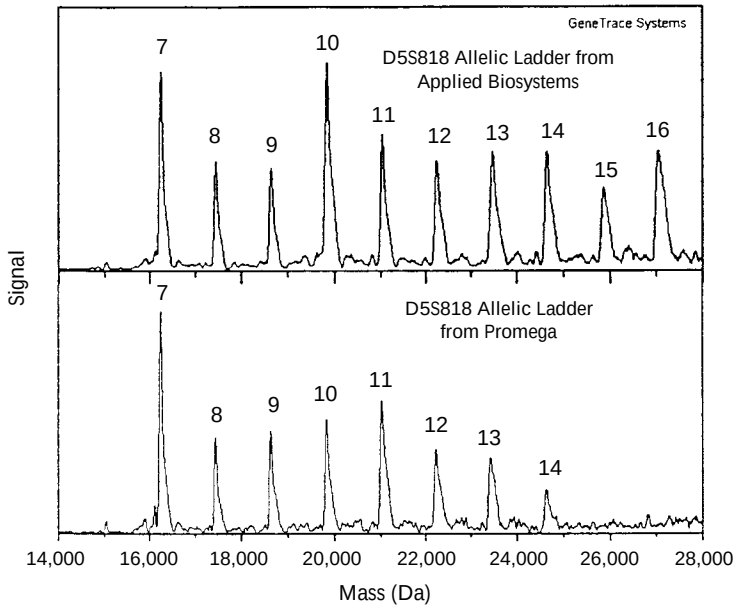


Exhibit 56. **Mass spectra of D5S818 allelic ladders from two manufacturers.** The Applied Biosystems D5S818 ladder contains 10 alleles (top panel); the Promega D5S818 ladder contains only 8 alleles (bottom panel). The GeneTrace primers bind internally to commercially available multiplex primers, and all alleles in the commercial allelic ladders are therefore amplified, demonstrating that the GeneTrace primers can amplify all common alleles for this particular STR locus.



Resolution

Dinucleotide repeats, such as CA repeats, require a resolution of at least 2 bp in order to resolve stutter products from the true allele or heterozygotes that differ by a single repeat. Trinucleotide and tetranucleotide repeats, with their larger repeat structure, are more easily resolved because there is a larger mass difference between adjacent alleles. However, the overall mass of the PCR product increases more rapidly with tri- or tetranucleotide repeats. For example, the repeat region for 40 GA repeats is 25,680 Da, while the mass of the repeat region quickly increases to 37,200 Da for 40 AAT repeats and 50,400 Da for 40 AATG repeats.

GeneTrace has demonstrated that a resolution of a single dinucleotide repeat (~600 Da) may be obtained for DNA molecules up to a mass of ~35,000 Da. This reduced resolution at higher mass presents a problem for polymorphic STR loci such as D18S51, D21S11, and FGA because single base resolution is often required to accurately call closely spaced alleles or to distinguish a microvariant containing a partial repeat from a full-length allele. These three STR loci also contain long alleles. For example, D21S11 has reported alleles of up to 38 repeats (mixture of TCTA and TCTG) in length, D18S51 up to 27 AGAA repeats, and FGA up to 50 repeats (mixture of CTTT and CTTC). Heterozygous FGA alleles that differed by only a single repeat were more difficult to genotype accurately than smaller sized STR loci due to poor resolution at masses greater than ~35,000 Da (see samples marked in red in exhibit 19).

The analysis of STR allelic ladders demonstrates that all alleles can be resolved for an STR locus. Allelic ladders from commercial kits were typically diluted 1:1000 with deionized water and then reamplified with the

GeneTrace primers that bound closer to the repeat region than the primers from the commercial kits. This reamplification provided PCR products for demonstrating that the needed level of resolution (i.e., distinguishing adjacent alleles) is capable at the appropriate mass range in the mass spectrometer as well as demonstrating that the GeneTrace primers amplify all alleles (i.e., no allele dropout from a null allele). A number of STR allelic ladders were tested in this fashion, including TH01 (exhibit 55); CSF1PO, TPOX, and VWA (exhibit 36); and D5S818 (exhibit 56). All tetranucleotide repeat alleles were resolvable in these examples, demonstrating 4 bp resolution, and TH01 single base pair resolution was seen between alleles 9.3 and 10.

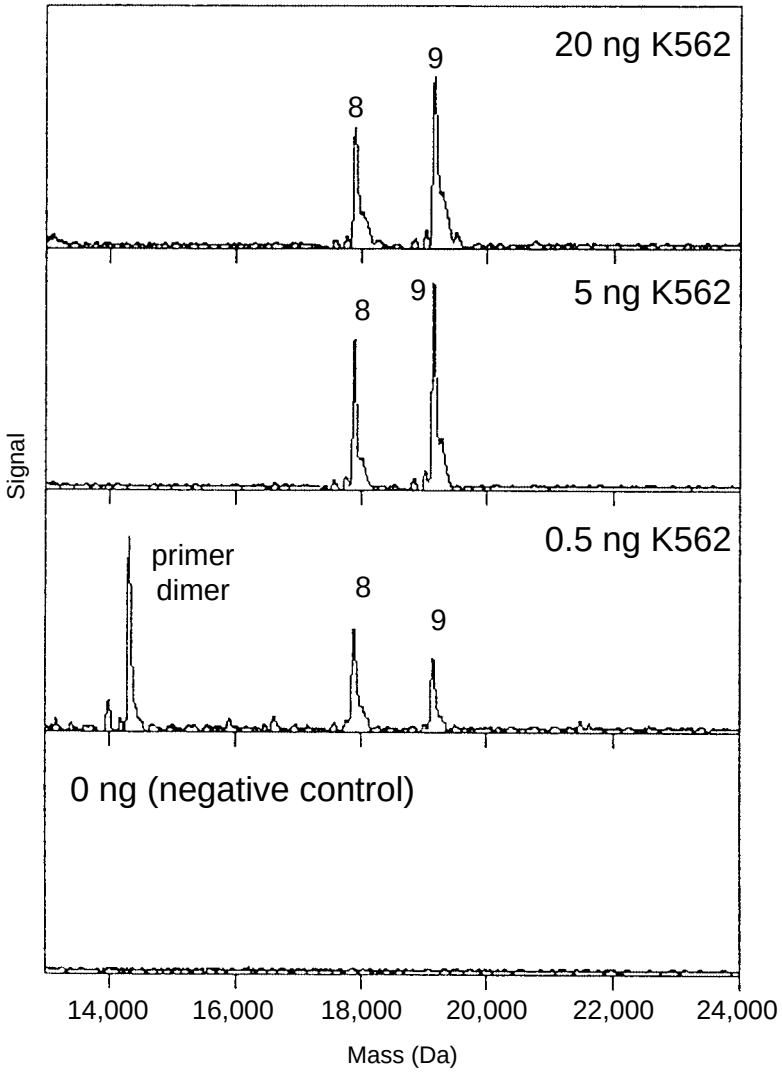
Sensitivity

To determine the sensitivity of GeneTrace's STR typing assay, TPOX primers were tested with a dilution series of K562 genomic DNA (20 ng, 10 ng, 5 ng, 2 ng, 1 ng, 0.5 ng, 0.2 ng, and 0 ng). Promega's Taq polymerase and STR buffer were used with 35 PCR cycles as described in the scope and methodology section. Peaks for the correct genotype (heterozygote 8,9) could be seen down to the lowest level tested (0.2 ng or 200 picograms), while the negative control was blank. Exhibit 57 contains a plot with the mass spectra for 20 ng, 5 ng, 0.5 ng, and 0 ng. While each PCR primer pair can exhibit a slightly different efficiency, human DNA down to a level of ~1 ng can be reliably PCR amplified and detected using mass spectrometry. GeneTrace's most recent protocol involved 40-cycle PCR and the use of TaqGold™ DNA polymerase, which should improve overall yield for STR amplicons. All of the samples tested from CDOJ were amplified with only 5 ng of DNA template and yielded excellent results (exhibits 12–19, 31, 39, 40, 43, 58, and 59). In terms of absolute sensitivity in the mass spectrometer, several hundred femtomoles of relatively salt-free DNA molecules were typically found necessary for detection. GeneTrace's PCR amplifications normally produced several picomoles of PCR product, approximately an order of magnitude more material than is actually needed for detection.

Mass accuracy and precision

Mass accuracy is an important issue for this mass spectrometry approach to STR genotyping, as a measured mass for a particular allele is compared with an ideal mass for that allele. Due to

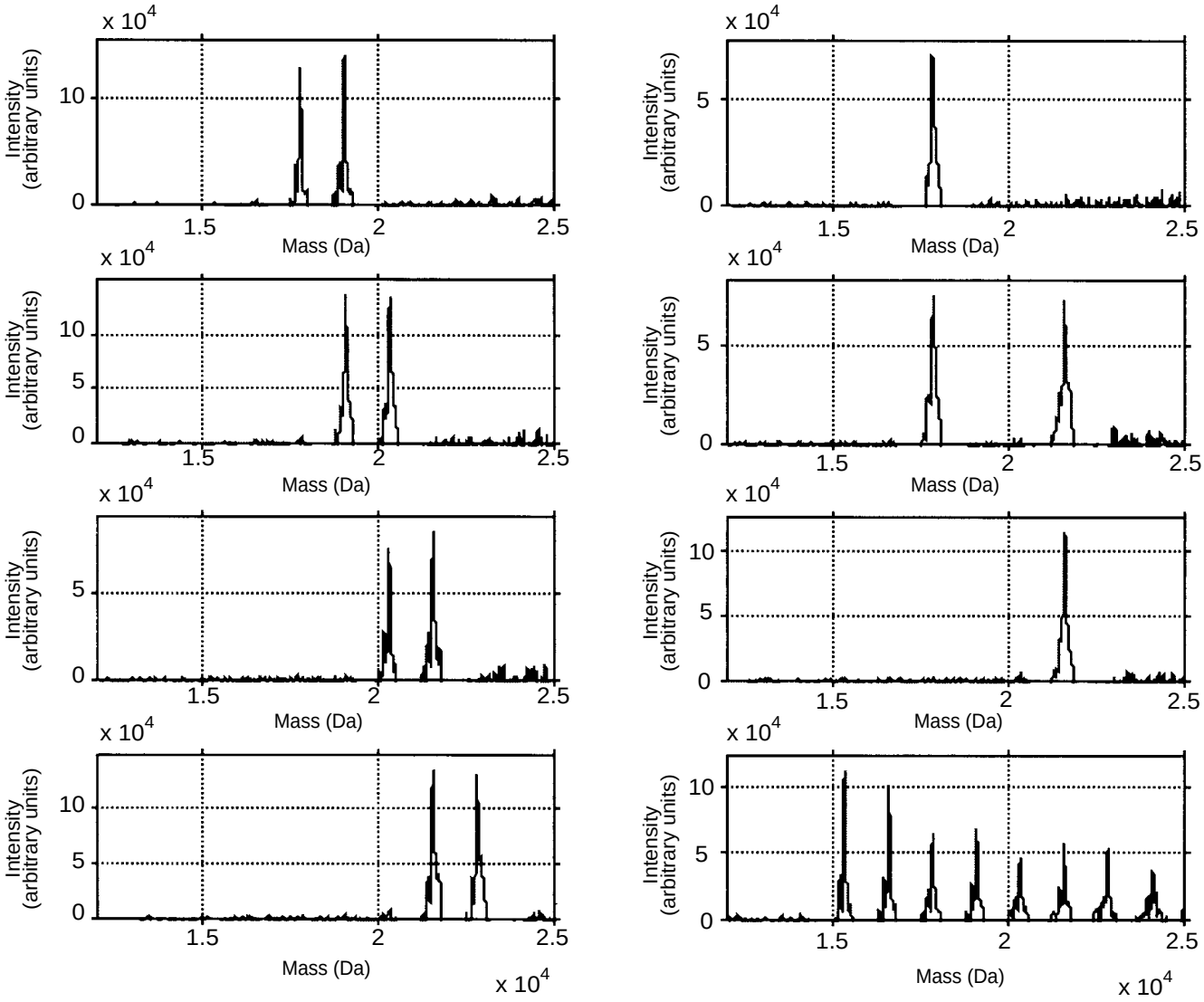
Exhibit 57. **Mass spectra of TPOX PCR products from various amounts of K562 DNA template material.** This sensitivity test demonstrates that DNA templates in the quantity range of 0.5–20 ng may be effectively amplified and detected by mass spectrometry.



the excellent accuracy of mass spectrometry, internal standards are not required to obtain accurate DNA sizing results as in gel or CE measurements (Butler et al., 1998). To make an inaccurate genotype call for a tetranucleotide repeat, the mass offset from an expected allele mass would have to be larger than 600 Da (half the mass of a ~1,200 Da repeat).

GeneTrace has observed mass accuracies on the order of 0.01 nucleotides (<3 Da) for STR allele measurements. However, under routine operation with GeneTrace's automated mass spectrometers, some resolution, sensitivity, and accuracy may be sacrificed compared with a research-grade instrument to deliver data at a high rate of speed. Almost all STR allele size measurements should be within ±200 Da, or a fraction of a single nucleotide, of the expected mass. Exhibit 60 illustrates that the precision and accuracy for STR measurements is good enough to make accurate genotyping calls with only a routine mass calibration, even when comparing data from the same samples collected months apart.

Exhibit 58. **Mass spectra of CDOJ samples amplified with TPOX primers.** From left side, top-to-bottom, followed by right side, top-to-bottom, sample genotypes are (8,9), (9,10), (10,11), (11,12), (8,8), (8,11), (11,11), and allelic ladder. The mass range shown here is 15,000–25,000 Da.



Precision is important for STR allele measurements in mass spectrometry because no internal standards are being run with each sample to make adjustments for slight variations in instrument conditions between runs. To demonstrate the excellent reproducibility of mass spectrometry, 15 mass spectra of a TPOX allelic ladder were collected. A table of the obtained masses for alleles 6, 7, 8, 9, 10, 11, 12, and 13 shows that all alleles were easily segregated and distinguishable (exhibit 61). Statistical analysis of the data found that the standard deviation about the mean for each allele ranged from 20 to 27 Da, or approximately 0.1% relative standard deviation (RSD). The mass between alleles is equal to the repeat unit, which in the case of TPOX is 1,260 Da for an AATG repeat (exhibit 62). Thus, each allele is easily distinguishable.

Measurements were made of the same DNA samples over a fairly wide timespan, revealing that masses can be remarkably similar, even when data points are recollected months later. Exhibit 60 compares 57 allele meas-

Exhibit 59. **Mass spectra of CDOJ samples amplified with TH01 primers.** From left side, top-to-bottom followed by right side, top-to-bottom, sample genotypes are (6,7), (6,8), (6,9.3), (6,10), (7,7), (7,7), (7,8), and (7,9.3). The split peaks for each allele result from partial adenylation—i.e., both ±A peaks are present. The mass range shown here is 12,000–22,000 Da.

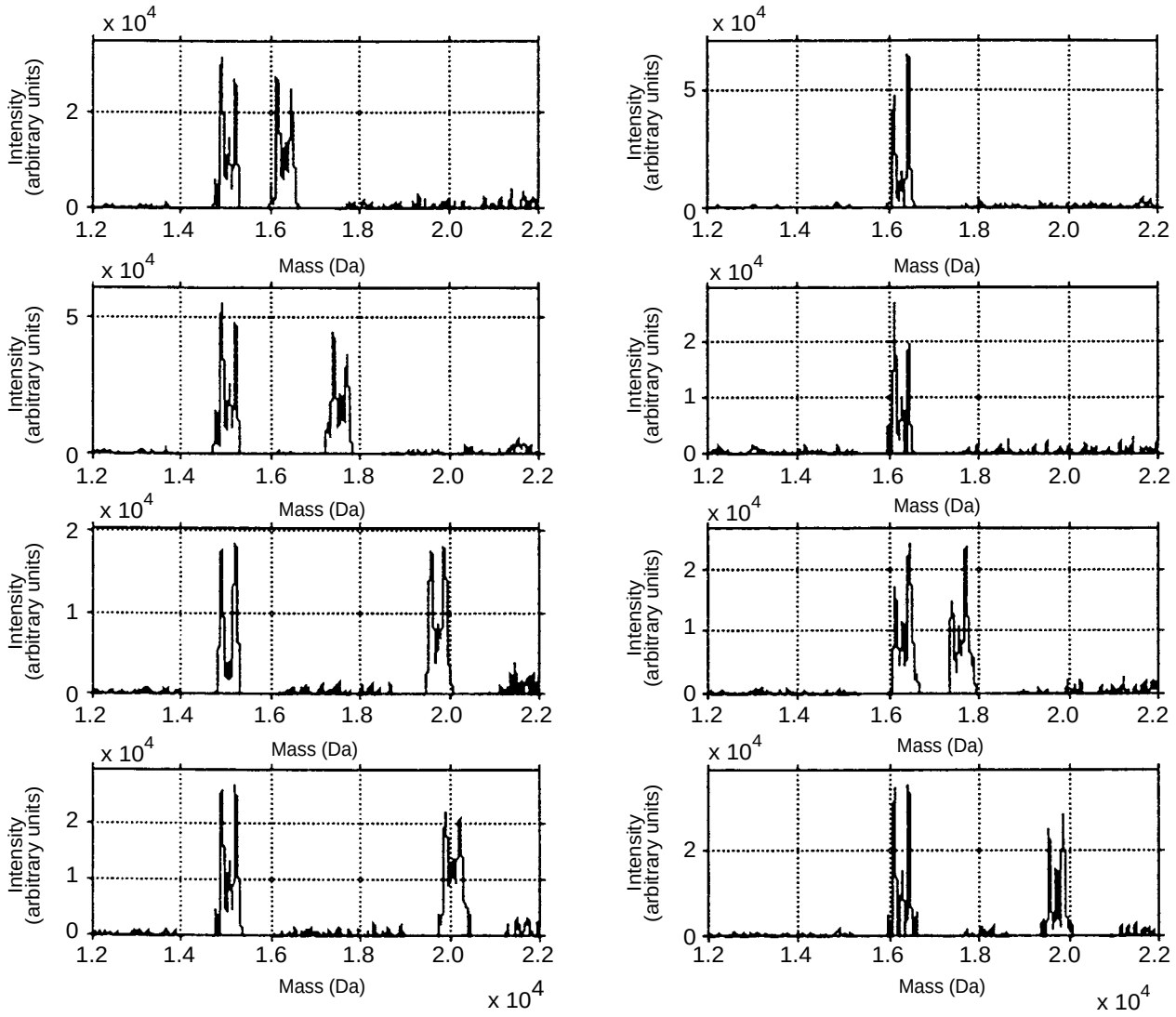
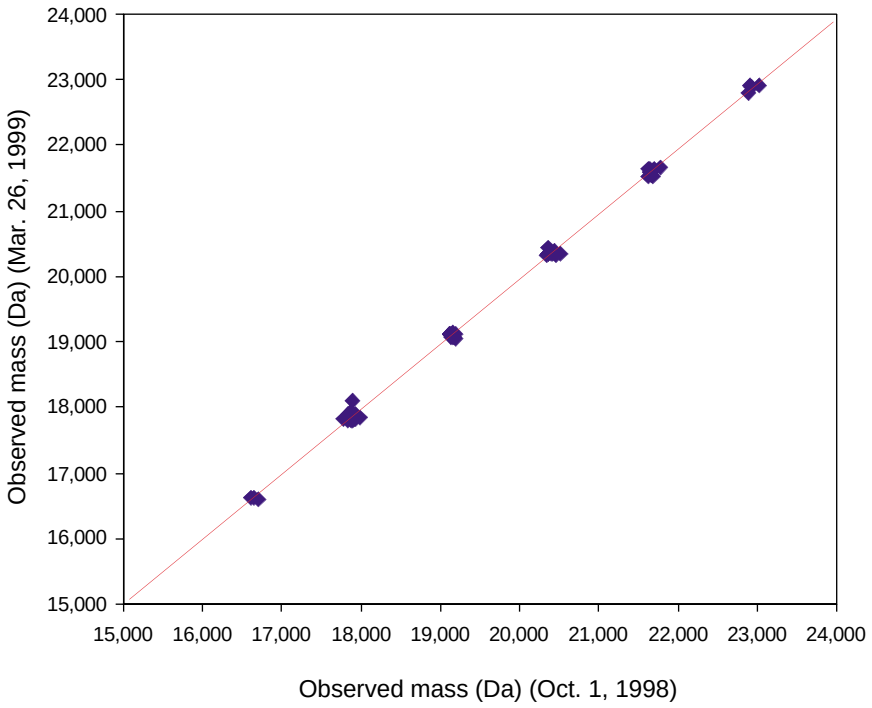


Exhibit 60. **Comparison of allele masses collected 6 months apart.** This plot compares 57 allele measurements of 6 different TPOX alleles. The ideal line is shown on the same plot to demonstrate how reproducible the masses are over time. The average standard deviation of allele mass measurements between these 2 data sets was 47 Da. This result further confirms that no allelic ladders or other internal DNA standards are needed to obtain accurate measurements with mass spectrometry.



urements from 6 different TPOX alleles collected 6 months apart. The first data set was collected on October 1, 1998, and the second data set on March 26, 1999. Amazingly enough, some of the alleles had identical measured masses, even though different mass calibration constants (and even different instruments) were used.

The bottom line is whether or not a correct genotype can be obtained using this new technology. Exhibit 63 compares the genotypes obtained using a conventional CE separation method and this mass spectrometry technique across 3 STR markers (D16S539, D8S1179, and CSF1PO) and indicates an excellent agreement between the methods. With the CDOJ samples tested, there was complete agreement on all observed genotypes for the STR loci CSF1PO, TH01, and D3S1358 as well as the sex-typing marker amelogenin (exhibits 12, 14–16). Some "gas-phase" dimers and trimers fell into the allele mass range and confused the calling for TPOX (exhibit 13) and D16S539 (exhibit 17) on several samples. Gas-phase dimers and trimers are assay artifacts that result from multiple excess primer molecules colliding in the gas phase and being ionized during the MALDI process. A mass offset plot like that shown in exhibit 46 can be used to detect these assay artifacts as they fall outside the tight grouping and inside the 300 Da window. With the CDOJ samples, D7S820 exhibited null alleles (exhibit 18) and FGA had some unique challenges due to its larger size, such as problems with resolution of closely spaced heterozygotes and poorer mass calibration since the measured alleles were further away from the calibration standards (exhibit 19). Thus, when the PCR situations such as null alleles are accounted for and smaller loci are used, this mass spectrometry method produces results comparable to traditional methods of STR genotyping.

Data collection speed

The tremendous speed advantage of mass spectrometry can be seen in exhibit 11. Over the course of this project, data collection speed increased by a factor of 10 from ~50 seconds/sample to less than 5 seconds/sample. This speed increase resulted from improved software and hardware on the automated mass spectrometers and from improved sample quality (i.e., better PCR conditions that yielded more product and improved sample cleanup that in turn yielded "cleaner" DNA). With data collection time around 5 seconds per sample, achieving sample throughputs of almost 1,000 samples per hour is possible, and 3,000–4,000 samples per system per day is reasonable when operating at full capacity. Sample backlogs could be erased rather rapidly with this kind of throughput. By way of comparison, it takes an average of 5 minutes to obtain each genotype (assuming a multiplex level of 6 or 7 STRs) using conventional CE methods (exhibit 11). Thus, the mass spectrometry method described in this study is two orders of magnitude faster in sample processing time than conventional techniques.

Exhibit 61. **Fifteen replicate analyses of a TPOX allelic ladder to measure mass precision and accuracy.** The precision was less than 30 Da for a single standard deviation, which corresponds to less than 0.1 nucleotide. The measured mass accuracy from the calculated expected allele masses averaged ~30 Da. Across the 8 alleles in the ladder, 120 data points are used to make this determination. All numbers are in Daltons (Da). Percentage error was calculated as (observed-expected)/ expected. This same data is also presented in histogram format; see figure 1 in Butler et al., 1998.

	Allele 6	Allele 7	Allele 8	Allele 9	Allele 10	Allele 11	Allele 12	Allele 13
Expected mass (Da)	15,345	16,605	17,865	19,125	20,385	21,644	22,904	24,164
Sample number								
1	15,346	16,623	17,903	19,130	20,388	21,623	22,860	24,074
2	15,387	16,667	17,901	19,129	20,387	21,639	22,893	24,114
3	15,372	16,629	17,887	19,143	20,400	21,615	22,877	24,091
4	15,385	16,653	17,903	19,163	20,384	21,642	22,903	24,076
5	15,388	16,642	17,898	19,155	20,337	21,654	22,870	24,111
6	15,336	16,600	17,857	19,105	20,362	21,599	22,832	24,064
7	15,388	16,637	17,894	19,131	20,383	21,635	22,904	24,110
8	15,363	16,618	17,872	19,129	20,368	21,604	22,853	24,087
9	15,365	16,628	17,891	19,150	20,385	21,620	22,892	24,087
10	15,373	16,638	17,892	19,136	20,394	21,631	22,878	24,085
11	15,383	16,640	17,896	19,152	20,387	21,621	22,884	24,129
12	15,388	16,648	17,912	19,172	20,388	21,623	22,850	24,149
13	15,407	16,660	17,941	19,208	20,425	21,674	22,944	24,148
14	15,410	16,659	17,930	19,174	20,425	21,666	22,893	24,132
15	15,390	16,648	17,915	19,157	20,423	21,636	22,897	24,126
Average mass	15,379	16,639	17,899	19,149	20,389	21,632	22,882	24,106
Std. dev.	20.2	17.8	20.6	24.7	23.7	21.0	27.2	27.3
%RSD	0.13	0.11	0.12	0.13	0.12	0.10	0.12	0.11
% error	0.22	0.21	0.19	0.13	0.02	-0.06	-0.10	-0.24
Obs-exp	33.7	34.3	34.5	23.9	4.1	-11.9	-22.0	-58.5

Exhibit 62. **Upper strand (TCAT repeat) and lower strand (AATG repeat) mass differences for the TH01 allelic ladder.** The upper strand was discernible from the lower strand due to the different sequence contents of the repeats. The STR repeat structure and nucleotide content can be seen using mass spectrometry. Note: The upper strand mass difference between alleles 9.3 and 10 is 306 Da, or a "T," and the lower strand mass difference between these same two alleles is 315 Da, or an "A." For more details, see Butler et al., 1998b.

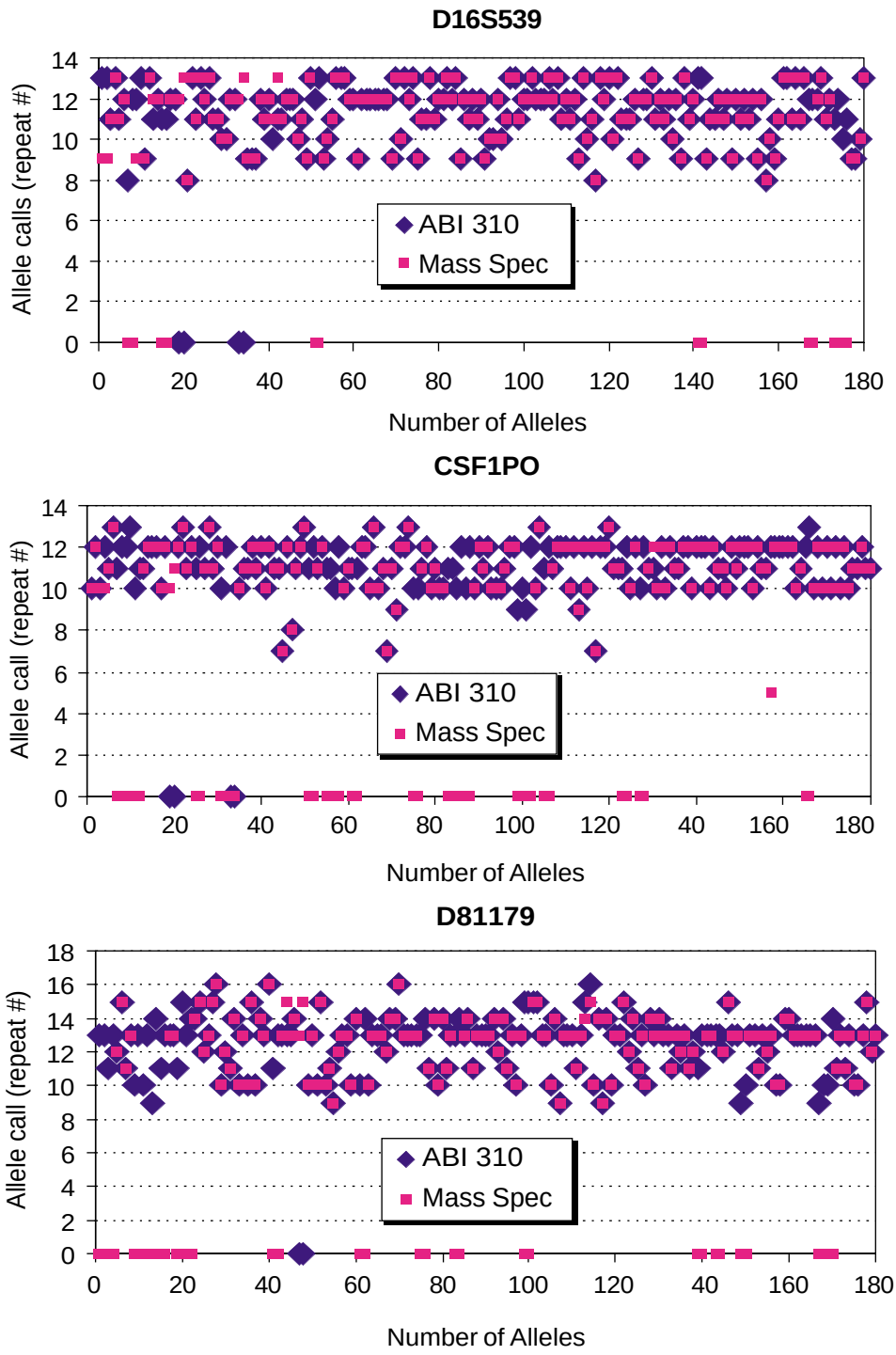
Upper Strand	Expected (Da)	Observed (Da)
Allele 5–6	1,211	1,210
Allele 6–7	1,211	1,211
Allele 7–8	1,211	1,215
Allele 8–9	1,211	1,215
Allele 9–9.3	907	915
Allele 9.3–10	304	306
Allele 9–10	1,211	1,221

Repeat = TCAT = 1,210.8 Da
= --CAT = 906.6 Da

Upper Strand	Expected (Da)	Observed (Da)
Allele 5–6	1,260	1,259
Allele 6–7	1,260	1,262
Allele 7–8	1,260	1,269
Allele 8–9	1,260	1,267
Allele 9–9.3	947	948
Allele 9.3–10	313	315
Allele 9–10	1,260	1,263

Repeat = AATG = 1,259.8 Da
= --ATG = 946.6 Da

Exhibit 63. **Comparison of ABI 310 and mass spectrometry allele calls for 90 CEPH/diversity samples.** Out of 1,080 possible allele calls with these 3 STR loci, there were 100 with no data collected (indicated as a "0" on the allele call axis), and only 12 calls differed between the two methods, or ~98% correlation.



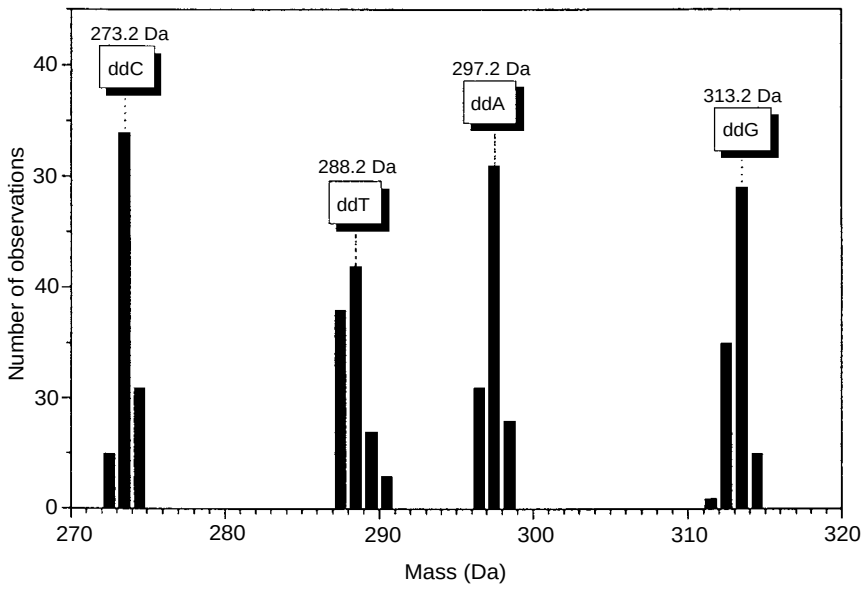
Results and Discussion of Multiplex SNPs

Work began on the development of multiplexed SNP assays in the summer of 1998 after notice that a second NIJ grant, Development of Multiplexed Single Nucleotide Polymorphism Assays from Mitochondrial and Y-Chromosome DNA for Human Identity Testing Using Time-of-Flight Mass Spectrometry, had been funded. Excellent progress was made toward the milestones on this grant, but the work not finished because this grant was prematurely terminated on the part of GeneTrace in the spring of 1999. The completed work focused on two areas: the development of a 10-plex SNP assay from the mtDNA control region using a single amplicon and the development of a multiplex PCR assay from Y-chromosome SNP markers that involved as many as 18 loci amplified simultaneously. This section describes the design aspects of multiplex PCR and SNP assays along with the progress made toward the goal of producing assays that would be useful for high-throughput screening of mitochondrial and Y-chromosome SNP markers.

The approach to SNP determination described here has essentially three steps: (1) PCR amplification, (2) phosphatase digestion, and (3) SNP primer extension. Either strand of DNA may be probed simultaneously in this SNP primer extension assay. PCR primers are designed to generate an amplicon that includes one or more SNP sites. The initial PCR reaction is performed with standard (unlabeled) primers. A phosphatase is then added following PCR to remove all remaining dNTPs so that they will not interfere with the single base extension reaction involving ddNTPs. These reactions can all be performed in the same tube or well in a sample tray. A portion of the phosphatase-treated PCR product is then used for the primer extension assay.

In the SNP primer extension assay, a special primer containing a biotin moiety at the 5'-end permits solid-phase capture for sample purification prior to mass spectrometry analysis. This primer hybridizes upstream of the SNP site with the 3'-end immediately adjacent to the SNP polymorphic site. A cleavable nucleotide near the 3'-end allows the 3'-end of the primer to be released from the immobilized portion and reduces the overall mass of the measured DNA molecule (exhibit 21) (Li et al., 1999). The complementary nucleotide(s) to the nucleotide(s) present at the SNP site is inserted during the extension reaction. In the case of a heterozygote, two extension products result. Only a single base is added to the primer during this process because only ddNTPs are used and the dNTPs left over from PCR are hydrolyzed with the phosphatase digestion step. If the extension reaction is not driven to completion (where the primer would be totally consumed), then both primer and extension product (i.e., primer plus single nucleotide) are present after the primer extension reaction. The mass difference between these two DNA oligomers is used to determine the nucleotide present at the SNP site. In the primer extension SNP assay, the primer acts as an internal standard and helps make the measurement more precise. A histogram of mass difference measurements across 200 samples (50 per nucleotide) is shown in exhibit 64. The ddT and ddA differ by only 9 Da and are the most difficult to resolve as heterozygotes or distinguish from one

Exhibit 64. **Histogram of mass difference measurements for 200 samples (50 for each ddN).** Expected masses for the dideoxynucleotides are 273.2 Da for ddC, 288.2 Da for ddT, 297.2 Da for ddA, and 313.2 Da for ddG. The overall mass precision with this set of samples was less than 2 Da. For more details, see Li et al., 1999.

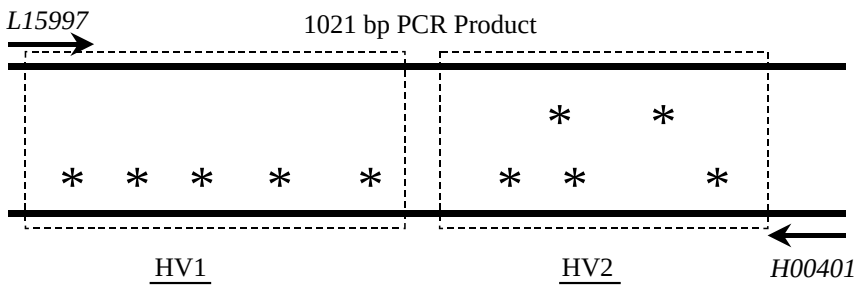


another in terms of mass. As reported in a recently published paper (Li et al., 1999), this approach has been used to reliably determine all four possible SNP homozygotes and all six possible heterozygotes.

Mitochondrial DNA Work

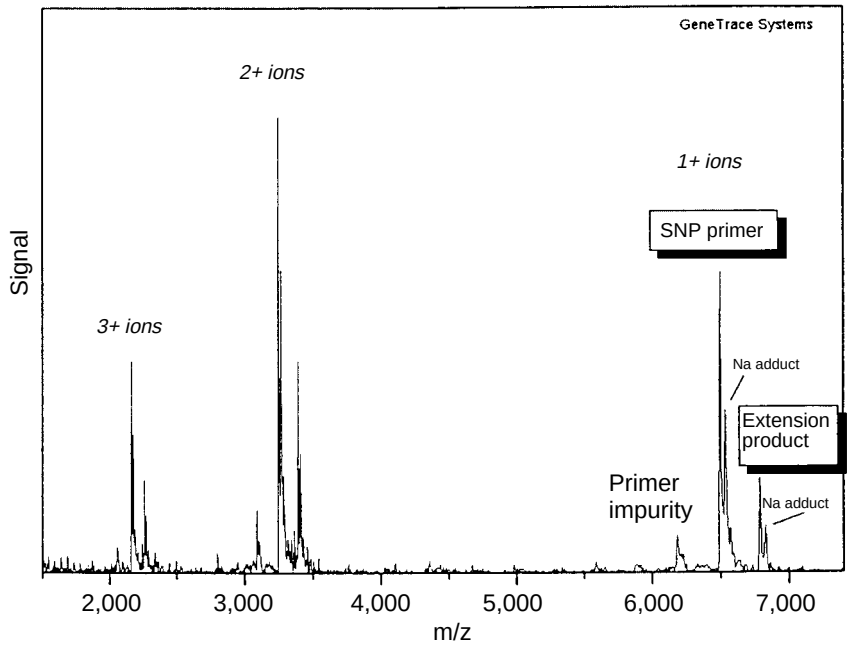
The control region of mtDNA, commonly referred to as the D-loop, is highly polymorphic and contains a number of possible SNP sites for analysis. MITOMAP, an internet database containing fairly comprehensive information on mtDNA, lists 408 polymorphisms over 1,121 nucleotides of the control region (positions 16020–576) that have been reported in literature (MITOMAP, 1999). However, many of these polymorphisms are rare and population specific. The present study focused on marker sets from a few dozen well-studied potential SNP sites. Special Agent Mark Wilson from the FBI Laboratory in Washington, D.C., who has been analyzing mtDNA for more than 7 years, recommended a set of 27 SNPs that would give a reasonable degree of discrimination and make the assay about half as informative as full sequencing. His recommended mtDNA sites were positions 16069, 16114, 16126, 16129, 16189, 16223, 16224, 16278, 16290, 16294, 16296, 16304, 16309, 16311, 16319, 16362, 73, 146, 150, 152, 182, 185, 189, 195, 198, 247, and 309. The underlined sites are those reported in a minisequencing assay developed by the Forensic Science Service (Tully et al., 1996). GeneTrace's multiplex SNP typing efforts began with 10 of the SNPs used in the FSS minisequencing assay, since those primer sequences had already been reported and studied together.

Exhibit 65. **Schematic representation of the mtDNA control region 10-plex SNP assay.** The asterisks represent the relative positions of the SNP sites and the strand that is probed.



HV1		HV2	
H16069	G or A	H00073	T or C
H16129	C or T	L00146	T or C
H16189	A or G	H00152	A or G
H16224	A or G	L00195	T or C
H16311	A or G	H00247	C or T

Exhibit 66. **SNP ions impacting multiplex design.** Doubly charged and triply charged ions from higher mass primers can interfere with singly charged ions of smaller mass primers if the multiplex is not well designed.



The reported FSS sequences were modified slightly by removing the poly(T) tail and converting the degenerate bases into the most common sequence variant (identified by examination of MITOMAP information at the appropriate mtDNA position). A cleavable base was also incorporated at varying positions in different primers so that the cleaved primers would be resolvable on a mass scale. Exhibit 26 lists the final primer set chosen for a 10-plex SNP reaction. Eight of the primers detected SNPs on the "heavy" GC-rich strand and two of them identified SNPs on the "light" AT-rich strand of the mtDNA control region (exhibit 65). Five of the primers annealed within hypervariable region I (HV1) and five annealed within hypervariable region II (HV2). All of the 10 chosen SNP sites were transitions of either A to G (purine-to-purine) or C to T (pyrimidine-to-pyrimidine) rather than transversions (purine-to-pyrimidine).

Besides primer compatibility (i.e., lack of primer dimer formation or hairpins), another important aspect of multiplex SNP primer design is the avoidance of multiple-charged ions. Doubly charged and triply charged ions of larger mass primers can fall within the mass-to-charge range of smaller primers. Depending on the laser energy used and matrix crystallization, the multiple-charged ions can be significantly abundant (exhibit 66). Primer impurities, such as n-1 failure products, can also impact how close together primers can be squeezed on a mass scale. These primer synthesis failure products will be ~300 Da smaller in mass than the full-length primer. Since an extension product ranges from 273 (ddC) to 313 Da (ddG) larger than the primer itself, a minimum of 650–700 Da is needed between adjacent primers (postcleavage mass) if primer synthesis failures exist to avoid any confusion in making the correct SNP genotype call.

Primer synthesis failure products were observed to become more prevalent for larger mass primers. Because resolution and sensitivity in the mass spectrometer decrease at higher masses, it is advantageous to keep the multiplexed primers in a fairly narrow mass window and as small as possible. The primers in this study ranged from 1,580 to 6,500 Da. Exhibit 23 displays the expected primer masses for the mtDNA SNP 10-plex along with their doubly and triply charged ions. The smallest four primers, in the mass range of 1,580–3,179 Da, had primer and extension masses that were similar to multiple-charged ions of larger primers. For example, in the bottom panel of exhibit 6, which shows the 10-plex primers, the doubly charged ion from MT4e (3,250 Da), which probes site L00195, fell very close to the singly charged ion from MT3' (3,179 Da), which probes site H16189. The impact of primer impurity products can also be seen in exhibit 6. An examination of the extension product region from primer MT7/H00073 (~6,200 Da) shows two peaks where only one was expected (top panel). The lower mass peak in the doublet is labeled as "+ddC" (6,192 Da), but the larger peak in the doublet was a primer impurity of MT4e/L00195 (6,232 Da). The mass difference between these two peaks was 40 Da or exactly what one would expect for a C/G heterozygote extension of primer MT7/H00073. Thus, to avoid a false positive, it was important to run the 10 primers alone as a negative control to verify any primer impurities.

To aid development of this multiplex SNP assay, large quantities of PCR product were produced from K562 genomic DNA (enough for ~320 reactions) and were pooled together so that multiple experiments would have the same starting material. With the K562 amplicon pool, the impact of primer concentration variation was examined without worrying about the DNA template as a variable. The K562 amplicon pool was generated using the PCR primers noted in exhibit 26, which produced a 1,021 bp PCR product that spanned the entire D-loop region (Wilson et al., 1995). Thus, all 10 SNP sites could be examined from a single DNA template.

Using ABI's standard sequencing procedures and dRhodamine dye-terminator sequencing kit, this PCR pool was sequenced to verify the identity of the nucleotide at each SNP site in the 10-plex. The sequencing primers were the same as those reported previously (Wilson et al., 1995). Identical results were obtained between the sequencing and the mass spectrometer, which verified the method (exhibit 6).

A variety of primer combinations and primer concentrations were tested on the way to obtaining results with the 10-plex. For example, a 4-plex and a 6-plex were developed first with primers that were further apart in terms of mass and, therefore, could be more easily distinguished. An early 6-plex was published in *Electrophoresis* (Li et al., 1999). Primer concentrations were balanced empirically by first running all primers at 10 pmol and then raising or lowering the amount of primer in the next set to obtain a good balance between those in the multiplex primer mix. In general, a higher amount of primer was required for primers of higher mass. However, this trend did not always hold true, probably because ionization efficiencies in MALDI mass spectrometry differ depending on DNA sequence content. The primer concentrations in the final "optimized" 10-plex ranged from 10 pmol for MT3' (3,179 Da) to 35 pmol for MT7 (5,891 Da). Primer extension efficiencies also varied

between primers, making optimization of these multiplexes rather challenging.

Originally, this study set out to examine 100 samples, but due to the early termination of the project, researchers were unable to run this multiplex SNP assay across a panel of samples to verify that it worked with more than one sample. Future work could include examination of a set of population samples and correlation to DNA sequencing results. Examination of the impact of SNPs that are close to the one being tested and that might impact primer annealing also needs to be done. In addition, more SNP sites can be developed and the multiplex could be expanded to include a larger number of loci.

Y-Chromosome Work

While the mtDNA work produced an opportunity to examine the mass spectrometry factors in developing an SNP multiplex, this work involved only a single DNA template with multiple SNP probes. A more common situation for multiplex SNP development is multiple DNA templates with one or more SNP per template. SNP sites may not be closely spaced along the genome and could require unique primer pairs to amplify each section of DNA. To test this multiplex SNP situation, researchers investigated multiple SNPs scattered across the Y chromosome. Through a collaboration with Dr. Oefner and Dr. Underhill, 20 Y-chromosome SNP markers were examined in this study. Dr. Oefner and Dr. Underhill have identified almost 150 SNP loci on the Y chromosome, some of which have been reported in the literature (Underhill et al., 1997). By examining an initial set of 20 Y SNPs and adding additional markers as needed, researchers attempted to develop a final multiplex set based on ~50 Y SNP loci. The collaboration provided detailed sequence information on bases around the SNP sites (typically several hundred bases on either side of the SNP site), which is important for multiplex PCR primer design. Dr. Oefner also provided a set of 38 male genomic DNA samples from various populations around the world for testing purposes.

The sequences were provided in two batches of 10 sequences each. In the first two sets, primer designs were attempted for a 9-plex PCR and a 17-plex PCR, respectively. Due to primer incompatibilities, it was impossible to incorporate all SNPs into each multiplex set. However, with a larger set of sequences to choose from, it is conceivable that much larger PCR multiplexes can be developed. According to Dr. Underhill's nomenclature, the first set of Y SNP markers included the following loci: M9 (C→G), M17 (1 bp deletion, 4Gs→3Gs), M35 (G→C), M42 (A→T), M45 (G–>A), M89 (C→T), M96 (G→C), M122 (T→C), M130 (C→T), and M145 (G→A). The second set of Y SNP markers contained these loci: M119 (A→C), M60 (1 bp insertion, a "T"), M55 (T→C),

Exhibit 67. **Multiplex PCR information for 17-plex PCR reaction**

Locus Name	Expected SNP	Primers Used	Primer Amounts	PCR Product Size (bp)
M3	C/T	F1u/R1u	0.4 pmole each	148 bp
M17	1 bp del	F1u/R1u	0.4 pmole each	149 bp
M13	G/C	F1u/R1u	0.4 pmole each	172 bp
M119	A/C	F1u/R1u	0.4 pmole each	188 bp
M2	A/G	F3u/R3u	0.4 pmole each	194 bp
M96	G/C	F1u/R1u	0.4 pmole each	198 bp
M122	T/C	F2u/R2u	0.4 pmole each	208 bp
M145	G/A	F1u/R1u	0.4 pmole each	218 bp
M45	G/A	F2u/R2u	0.4 pmole each	230 bp
M35	G/C	F1u/R5u	0.4 pmole each	233 bp
M9	C/G	F1u/R1u	0.4 pmole each	243 bp
M55	T/C	F1u/R1u	0.4 pmole each	247 bp
M60	1 bp ins	F1u/R1u	0.4 pmole each	263 bp
M89	C/T	F4u/R4u	0.4 pmole each	275 bp
M42	A/T	F1u/R1u	0.4 pmole each	300 bp
M67	A/T	F1u/R1u	0.4 pmole each	314 bp
M69	T/C	F1u/R1u	0.4 pmole each	326 bp
M26	G/C	F1u/R1u	0.4 pmole each	333 bp
M130	C/T	F1u/R1u	Included in 9-plex but not 17-/18-plex PCR	155 bp

M20 primers are not compatible with the other loci in the multiplex.

PCR Mix: 5 mM $MgCl_2$, 1X PCR buffer II, 250 µM dNTPs, 2 U TaqGold, 40 pmol univ-F primer, 40 pmol univ-R primer, and 0.4 pmole each locus-specific primer pair in 20 µL volume

Thermal Cycling: 95 ^{o}C for 10 min; 50 cycles at 94 ^{o}C for 30 sec, 55 ^{o}C for 30 sec, 68 ^{o}C for 60 sec, 72 ^{o}C for 5 min, and 4 ^{o}C forever (~3 hr total)

M20 (A→G), M69 (T→C), M67 (A→T), M3 (C→T), M13 (G→C), M2 (A→G), and M26 (G→A).

Multiplex PCR primers were designed with a UNIX version of Primer 3 (release 0.6) (Rozen et al., 1998) that was adapted at GeneTrace by Nathan Hunt to utilize a mispriming library and Perl scripts for input and export of SNP sequences and primer information, respectively. The PCR primer sequences produced by Hunt's program are listed in exhibit 27. The universal tags attached to each primer sequence aid in multiplex compatibility (Shuber et al., 1995). This tag added 23 bases to the 5'-end of the forward primers and 24 bases to the 5'-end of the reverse primers and, therefore, increased the overall length of PCR products by 47 bp. The addition of the universal tag makes multiplex PCR development much easier and reduces the need to empirically adjust primer concentrations to balance PCR product quantities obtained from multiple loci (Ross et al., 1998).

To compare the amplicon yields from various loci amplified in the multiplex PCR, the product sizes were selected to make them resolvable by CE separation. Thus, the PCR product sizes ranged from 148 bp to 333 bp (exhibit 67) using the primers listed in exhibit 27. To make sure that each primer pair worked, each marker was amplified individually as well as in the multiplex set using the same concentration of PCR primers. A substantial amount of primers remaining after PCR indicated that the PCR efficiency was lower for that particular marker (exhibit 68). Researchers were able to demonstrate male-specific PCR with a 17-plex set of PCR primers. The male test sample AM209 from an Amish CEPH family (exhibit 25) produced amplicons for 17 Y SNP loci, while K562 genomic DNA yielded no detectable PCR product because it is female DNA and, therefore, does not contain a Y chromosome (exhibit 7).

SNP primers were designed and synthesized for probing the SNP sites either in a singleplex (exhibit 69) or a multiplex (exhibit 70) format. Some additional SNP primers and multiplex PCR primers were also designed for testing 12 autosomal SNPs throughout the human genome with the hope of comparing the informativeness of SNPs to STRs (exhibit 71). Analysis of these same 12 SNPs was recently demonstrated in a multiplex PCR and SNP assay by PerSeptive Biosystems (Ross et al., 1998).

Optimal SNP markers for identity testing typically have allele frequencies of 30–70% in a particular human population. By way of comparison, highly polymorphic STRs can have 10–15 or more alleles with allele frequencies below 15% (i.e., more alleles and lower allele frequencies). The characteristics of STR and SNP markers are compared in exhibit 72. SNPs have the capability of being multiplexed to a much higher level than STRs; however, more SNP markers are required for the same level of discrimination compared with STRs. Only time will tell what role new SNP markers will have in human identity testing.

Exhibit 68. **CE electropherograms showing a multiplex PCR sample compared with individual PCR reactions.** The M67 primers are more efficient as fewer primers remain following the PCR reaction.

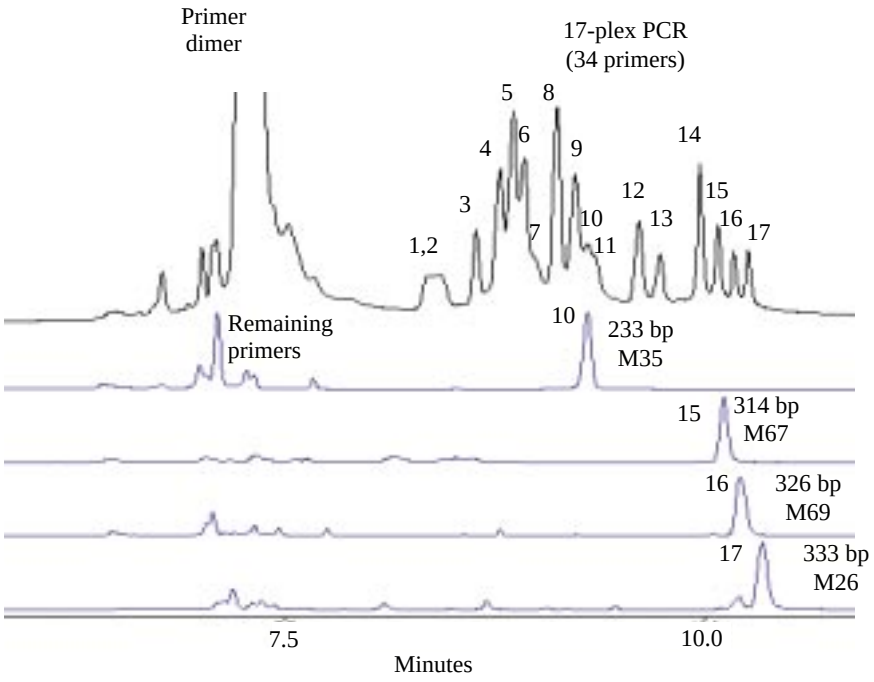


Exhibit 69. **Primers for testing Y-chromosome SNP markers in singleplexes.** Primers were designed automatically with UNIX SNP probe scripts written by Nathan Hunt but have not yet been tested.

Primer Name	Primer Sequence (5'→3')	Cleavage Mass (Da)	Expected SNP
M145-P1	b-CTTGCCTCCACGAC(T)TTCCT	1,491	A/G
M35-P1	b-CGGAGTCTCTGCC(T)GTGTC	1,556	C/G
M9-P1	b-AACGGCCTAAGATGG(T)TGAAT	1,564	C/G
M26-P1	b-AGGCCATTCAGTG(T)TCTCTG	1,820	C/G
M67-P1	b-TTGTTCGTGGACCCC(T)CTATAT (overlaps PCR reverse primer)	1,828	A/T
M45-P1	b-CCTCAGAAGGAGC(T)TTTTGC	1,835	C/T
M145-P2	b-GATTAGGCTAAGGC(T)GGCTCT	1,845	C/T
M119-P1	b-TTCCAATTCAGCA(T)ACAGGC	1,863	T/G
M55-P1	b-GCCCCTGGATGGTT(T)AAGTTA	1,877	C/T
M20-P1	b-ACCAACTGTGGAT(T)GAAAAT (no PCR primers designed)	1,886	A/G
M122-P1	b-TCAGATTTTCCCC(T)GAGAGC	1,903	A/G
M96-P1	b-TTGGAAAACAGGTCTC(T)CATAATA	2,150	C/G
M69-P1	b-GAGGCTGTTTACAC(T)CCTGAAA	2,151	C/T
M130-P1	b-GGGCAATAAACCT(T)GGATTTC (overlaps PCR forward primer)	2,173	C/T
M42-P1	b-CACCAGCTCTCTTTTTCAT(T)ATGTAGT	2,198	A/T
M89-P1	b-CAACTCAGGCAAAG(T)GAGAGAT (overlaps PCR reverse primer)	2,232	A/G
M3-P1	b-GGGTCACCTC(T)GGGACTGA	2,537	A/G
M2-P1	b-CCTTTATCC(T)CCACAGATCTCA (overlaps PCR reverse primer)	3,636	C/T
M13	Not designed		C/G
M60	Not designed (1bp insertion)		ins
M17	Not designed (1bp deletion)		del

Exhibit 70. **Y SNP multiplex primer information.** Primers were designed manually for multiplex SNP assay (9-plex) with nonoverlapping masses.

Primer Name	Primer Sequence (5'→3')	Cleaved Mass (Da)	Expected SNP
Y1 (M42b-a)	b-CCAGCTCTCTTTTTCATTA(T)GTAGT	1,580	T/A
Y2 (M96t-b)	b-CTTGGAAAACAGGTCTC(T)CATAATA	2,150	C/G
Y3 (M35b-c)	b-TTCGGAGTCTC(T)GCCTGTGTC	2,768	C/G
Y4 (M130b-b)	b-CCTT(T)CCCCTGGGCAG	3,380	C/T
Y5 (M145b-b)	b-GATTAGGC(T)AAGGCTGGCTCT	3,723	A/G
Y6 (M122t-c)	b-TAGAAAAGCAAT(T)GAGATACTAATTCA	4,333	C/T
Y7 (M45t-d)	b-AAATTGGCAG(T)GAAAAATTATAGATA	4,694	A/G
Y8 (M9b-c)	b-ACATGTCTAAA(T)TAAAGAAAAATAAAGAG	5,354	C/G
Y9 (M89t-e)	b-CTTCC(T)AAGGTTATGTACAAAAATCT	6,210	C/T

Exhibit 71. **Human autosomal SNP markers designed for testing NIH diversity panel**

SNP markers are not designed for multiplexing.

SNP Primer Name	Primer Sequence (5'→3')	Cleavage Mass (Da)	Expected (SNP)
C6-P1	b-GGGGACAGCCA(T)GCACTG	1,854	A/C
A2M-P1	b-GAAACACAGCAGCTTAC(T)CCAGAG	1,863	A/G
LDLR-P1	b-CCTATGACACCGTCA(T)CAGCAG	1,863	A/G
IL1A-P4	b-TTTTAGAAATCATCAAGCC(T)AGGTCA	1,878	T/G
CD18-P2	b-GGACATAGTGACCG(T)GCAGGT	1,894	C/T
IGF2-P1	b-CCACCTGTGATT(T)CTGGGG	1,910	C/T
ALDOB-P1	b-CGGGCCAAGAAGG(T)ATCTACC	2,102	A/G
PROS1-P1	b-CATAATGATATTAGAGCTCAC(T)CATGTCC	2,118	A/G
NF1-P1	b-CGATGGTTGTATTTGTCACCA(T)ATTAATT	2156	A/G
AT3-P0	b-ATCTCCA(T)GGGCCCAGC	2,787	C/T
CYP2D6-P1	b-GCAGCTTCAATGA(T)GAGAACCTG	2,810	C/T
LIPC-P0	b-AACATGGCT(T)CGAGAGAGTTG	3,483	A/C

Multiplex PCR primers for 12-plex PCR (not all amplicons are resolvable by CE). Universal primer sequences are in color.

Primer Name	Primer Sequence (5'→3')	PCR Product Size
AT3-F1u	ATT TAG GTG ACA CTA TAG AAT ACT GAG ACC TCA GTT TCC TCT TCT G	**159 bp**
AT3-R1u	TAA TAC GAC TCA CTA TAG GGA GAC CCT GGT CCC ATC TCC TCT AC	
C6-F1u	ATT TAG GTG ACA CTA TAG AAT ACA TCT GTC TTG CGT CCC AGT C	**160 bp**
C6-R1u	TAA TAC GAC TCA CTA TAG GGA GAC TCT TGC AGT CAG CCT CTT CA	
IGF2-F1u	ATT TAG GTG ACA CTA TAG AAT ACA GTC CCT GAA CCA GCA AAG A	**163 bp**
IGF2-R1u	TAA TAC GAC TCA CTA TAG GGA GAC TTT TCG GAT GGC CAG TTT AC	
LIPC-F1u	ATT TAG GTG ACA CTA TAG AAT ACA ACA CAC TGG ACC GCA AAA G	**173 bp**
LIPC-R1u	TAA TAC GAC TCA CTA TAG GGA GAC ACC CAG GCT GTA CCC AAT TA	
NF1-F1u	ATT TAG GTG ACA CTA TAG AAT ACA AGG AGC AAA CGA TGG TTG TA	**181 bp**
NF1-R1u	TAA TAC GAC TCA CTA TAG GGA GAC TAG GTG GCT GCA AGG TAT CC	
LDLR-F5u	ATT TAG GTG ACA CTA TAG AAT ACC CAC GGC GTC TCT TCC TAT	**181 bp**
LDLR-R5u	TAA TAC GAC TCA CTA TAG GGA GAC TGG TAT CCG CAA CAG AGA CA	
CYP2D6-F1u	ATT TAG GTG ACA CTA TAG AAT ACG GTG CAG AAT TGG AGG TCA T	**182 bp**
CYP2D6-R1u	TAA TAC GAC TCA CTA TAG GGA GAC AGA ACA GGT CAG CCA CCA CTA	
CD18-F2u	ATT TAG GTG ACA CTA TAG AAT ACA TCC AGG AGC AGT CGT TTG T	**193 bp**
CD18-R2u	TAA TAC GAC TCA CTA TAG GGA GAC ATG CCG CAC TCC AAG AAG	
ALDOB-F1u	ATT TAG GTG ACA CTA TAG AAT ACC ACA TTT GGG GCT TGA CTT T	**231 bp**
ALDOB-R1u	TAA TAC GAC TCA CTA TAG GGA GAC TCC TTC AGT CTC CTG TCA TCA A	
A2M-F1u	ATT TAG GTG ACA CTA TAG AAT ACC TCT GCC ATG CAA AAC ACA C	**247 bp**
A2M-R1u	TAA TAC GAC TCA CTA TAG GGA GAC AAC ATT CAA GTT TCC CTT ACT CAA	
PROS1-F1u	ATT TAG GTG ACA CTA TAG AAT ACT AAT GGC TGC ATG GAA GTG A	**292 bp**
PROS1-R1u	TAA TAC GAC TCA CTA TAG GGA GAC CCA GGA AAG GAC CAC AAA AT	
IL1A-F1u	ATT TAG GTG ACA CTA TAG AAT ACT TTG CTT CCT CAT CTG GAT TG	**324 bp**
IL1A-R1u	TAA TAC GAC TCA CTA TAG GGA GAC AGC AGC CGT GAG GTA CTG AT	

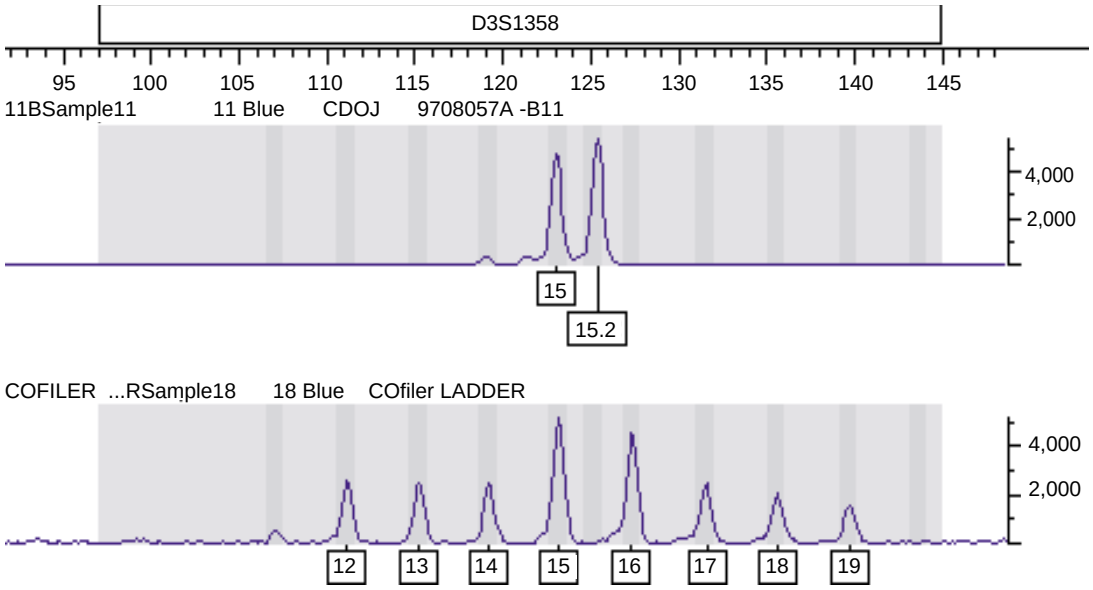
Exhibit 72. **Characteristics of STR and SNP markers.** SNPs are more common in the human genome than STRs but are not as polymorphic.

Characteristics	Short Tandem Repeats (STRs)	Single Nucleotide Polymorphisisms (SNPs)
Occurrence in human genome	~1 in every 15 kb	~1 in every 1 kb
General informativeness	High	Low (20–30% as informative as STRs)
Marker type	Di-, tri-, tetranucleotide repeat markers	Biallelic markers
Number of alleles per marker	Typically >5	Typically 2
Current detection methods	Gel/capillary electrophoresis	Microchip hybridization
Multiplex capability (fluorescence)	>10 markers with multiple spectral channels	Potentially thousands on microchip
Heterozygote resolution (fluorescence)	Mobility differences between alleles	Spectral differences between labeled nucleotides
Mass spectrometry measurement	Mass measurement of PCR-amplified allele(s)	Mass difference between primer and extension product

Exhibit 73. **STR genotypes for standard DNA templates K562, AM209, and UP006 obtained using AmpF1STR® ProfilerPlus™ and AmpF1STR® COfiler™ fluorescent STR kits.** These samples were the primary controls used for testing of newly developed primer sets. Alleles were not always well balanced for the K562 cell line DNA due to possible chromosome imbalances in the original sample. For example, the STR marker D21S11 produced three balanced alleles, most likely because three chromosome 21s were present in the K562 genomic DNA. In addition, the allele 24 peak ("24w" = "weak") for the FGA locus in K562 is only one-third the height of the allele 21 peak probably because there are more copies of the chromosome 4 on which the allele with 21 repeats resides. K562 genomic DNA came from Promega Corporation and AM209 came from CEPH pedigree 884 (Amish family). UP006 is an anonymous population sample of European origin purchased from Bios Laboratory.

STR locus	K562	AM209	UP006
TH01	9.3,9.3	6,7	7,9.3
TPOX	8,9	8,9	8,8
CSF1PO	9,10	10,12	11,14
D8S1179	12,12	11,13	13,13
D21S11	29,30,31	30,30	28,31
D18S51	15,16	12,12	13,13
D3S1358	16,16	15,18	15,15
VWA	16,16	18,18	16,17
FGA	21,24w	20,25	19,22
D16S539	11,12	11,12	12,13
D5S818	11,12	11,12	10,11
D13S317	8,8	14,14	11,14
D7S820	9,11	9,10	10,12
Amelogenin	X,X	X,Y	X,Y

Exhibit 74. **An ABI 310 CE electropherogram showing a heterozygous sample with a 15.2 microvariant at STR locus D3S1358.** A high degree of resolution is often necessary to resolve two closely spaced alleles in which one of them is a microvariant. In this case, the two alleles differ by only 2 bp even though the core repeat is a tetranucleotide. In the comparison with STR data collected by CDOJ, this was the only sample where a different result was obtained between our analysis and that performed by CDOJ on the same STR markers. CDOJ reported 14,15 for this sample.



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PUBLISHED PAPERS AND PRESENTATIONS

From 1997 to 1999, six publications resulted from the work funded by NIJ, and at least one more manuscript is in preparation. All articles were published in journals or for conference proceedings that are accessible and frequented by forensic DNA scientists to ensure proper dissemination of the information.

Butler, J.M., J. Li, T.A. Shaler, J.A. Monforte, and C.H. Becker. 1998. Reliable genotyping of short tandem repeat loci without an allelic ladder using time-of-flight mass spectrometry. *International Journal of Legal Medicine* 112 (1): 45–49.

Butler, J.M., J. Li, J.A. Monforte, C.H. Becker, and S. Lee. 1998. Rapid and automated analysis of short tandem repeat loci using time-of-flight mass spectrometry. In *Proceedings of the Eighth International Symposium on Human Identification 1997.* Madison, WI: Promega Corporation, 94–101.

Butler, J.M., K.M. Stephens, J.A. Monforte, and C.H. Becker. 1999. High-throughput STR analysis by time-of-flight mass spectrometry. In *Proceedings of the Second European Symposium on Human Identification 1998.* Madison, WI: Promega Corporation, 121–130.

Butler, J.M., and C.H. Becker. 1999. High-throughput genotyping of forensic STR and SNP loci using time-of-flight mass spectrometry. In *Proceedings of the Ninth International Symposium on Human Identification 1998.* Madison, WI: Promega Corporation, 43–51.

Li, J., J.M. Butler, Y. Tan, H. Lin, S. Royer, L. Ohler, T.A. Shaler, J.M. Hunter, D.J. Pollart, J.A. Monforte, and C.H. Becker. 1999. Single nucleotide polymorphism determination using primer extension and time-of-flight mass spectrometry. *Electrophoresis* 20:1258–1265.

Butler, J.M. 1999. STR analysis by time-of-flight mass spectrometry. *Profiles in DNA* 2(3): 3–6.

In addition, one patent was submitted based on work funded by NIJ. This patent describes the PCR primer sequences used to generate smaller amplicons for 33 different STR loci along with representative mass spectrometry results. The sequences for multiple cleavable primers are also described, although this proprietary chemistry is the subject of U.S. Patent 5,700,642, which was issued in December 1997. The process of multiplexing STR loci by interleaving the alleles on a compressed mass scale is also claimed by U.S. Patent 6,090,558 (Butler et al., 2000).

During the course of this NIJ grant, research findings were presented to the forensic DNA community at the following scientific meetings:

- Eighth International Symposium on Human Identification (September 20, 1997)
- San Diego Conference, Nucleic Acid Technology: The Cutting Edge of Discovery (November 7, 1997)
- NIJ Research Committee (February 8, 1998)
- American Academy of Forensic Sciences (February 13, 1998)
- Southwest Association of Forensic Scientists DNA training workshop (April 23, 1998)
- California Association of Criminalists DNA training workshop (May 6, 1998)
- National Conference on the Future of DNA (May 22, 1998)
- Florida DNA Training Session (May 22, 1998)
- American Society of Mass Spectrometry (June 4, 1998)
- Second European Symposium on Human Identification (June 12, 1998)
- IBC DNA Forensics Meeting (July 31, 1998)
- Ninth International Symposium on Human Identification (October 8, 1998)
- Fourth Annual CODIS User's Group Meeting (November 20, 1998)
- NIJ Research Committee (February 15, 1999)

In addition, the authors participated in NIJ's "Technology Saves Lives" Technology Fair on Capitol Hill in Washington, D.C., March 30–31, 1998, an event that provided excellent exposure for NIJ to Congress. Here, one of the DNA sample preparation robots was demonstrated in the lobby of the Rayburn Building. In September 1998, an 11-minute video was also prepared to illustrate some of the advantages of mass spectrometry for high-throughput DNA typing.

About the National Institute of Justice

NIJ is the research and development agency of the U.S. Department of Justice and is the only Federal agency solely dedicated to researching crime control and justice issues. NIJ provides objective, independent, nonpartisan, evidence-based knowledge and tools to meet the challenges of crime and justice, particularly at the State and local levels. NIJ's principal authorities are derived from the Omnibus Crime Control and Safe Streets Act of 1968, as amended (42 U.S.C. §§ 3721–3722).

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In partnership with others, NIJ's mission is to prevent and reduce crime, improve law enforcement and the administration of justice, and promote public safety. By applying the disciplines of the social and physical sciences, NIJ—

- **Researches** the nature and impact of crime and delinquency.
- **Develops** applied technologies, standards, and tools for criminal justice practitioners.
- **Evaluates** existing programs and responses to crime.
- **Tests** innovative concepts and program models in the field.
- **Assists** policymakers, program partners, and justice agencies.
- **Disseminates** knowledge to many audiences.

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NIJ is committed to five challenges as part of its strategic plan: 1) **rethinking justice** and the processes that create just communities; 2) **understanding the nexus** between social conditions and crime; 3) **breaking the cycle** of crime by testing research-based interventions; 4) **creating the tools** and technologies that meet the needs of practitioners; and 5) **expanding horizons** through interdisciplinary and international perspectives. In addressing these strategic challenges, the Institute is involved in the following program areas: crime control and prevention, drugs and crime, justice systems and offender behavior, violence and victimization, communications and information technologies, critical incident response, investigative and forensic sciences (including DNA), less-than-lethal technologies, officer protection, education and training technologies, testing and standards, technology assistance to law enforcement and corrections agencies, field testing of promising programs, and international crime control. NIJ communicates its findings through conferences and print and electronic media.

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